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Coliforms in Drinking Water Emanating from Redwood Reservoirs

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Coliforms in Drinking Water Emanating from Redwood Reservoirs

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16. ABSTRACT

Coliforms of the genus Klebsiella were found in many finished drinking water supplies which used reservoirs constructed from redwood. Coliform contamination was associated with slime which accumulated on some stave surfaces below the water line on the tank interior. Based on the results of field surveys, design modifications were made on an experimental 1,000 gallon reservoir in order to control coliform contamination. Coliforms were not found when free chlorine residuals of 0.3 to 0.4 ppm were maintained in the tank water. Poor water mixing and retention times of 7 days or longer made it difficult to maintain a free residual due to the chlorine demand of the wooden reservoir. Minimum retention times (less than 7 days) and adequate water mixing (no common inlet/outlet) can be achieved by proper choice of reservoir size and regulation of float levels. In the absence of free chlorine residuals, Klebsiella are able to multiply on water soluble nutrients leached from the redwood. Cyclitols, comprising 4.5% redwood dry weight, are the nutrients which uniquely support the growth of Klebsiella and Enterobacter in the redwood environment. Information in this report will be of interest to technically oriented personnel in water supply and treatment and environmental microbiologists.

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COLIFORMS IN DRINKING WATER EMANATING FROM REDWOOD RESERVOIRS

by

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This project provided data to formulate recommendations on the significance and control of Klebsiella pneumoniae and other coliform organisms which are present in drinking water emanating from redwood storage tanks.

Francis T. Mayo, Director
Municipal Environmental Research
Laboratory

ABSTRACT

Klebsiella pneumoniae and related biotypes were often encoutered in finished drinking water supplies stored in redwood reservoirs. Biochemical tests indicated no significant difference between isolates from drinking water and those from clinical infections in man and animals. Approximately 17% of the *Klebisella* associated with redwood are fecal coliform positive and are indistinguishable in this test from isolates of *E. coli*. Multiple resistance to antibiotics was occassionally found among *Klebsiella* associated with redwood and these isolates could serve as donors in the transfer of antibiotic resistance to *E. coli* and to other *Klebsiella*. Comparative pathogenicity experiments by LD_{50} levels for mice illustrated that isolates of redwood associated *Klebsiella* have mean LD_{50} values indistinguishable from known human and animal pathogenic isolates. The mean LD_{50} for *E. coli* of human clinical origin is not significantly different from *Klebsiella*. However, *E. coli* isolates from drinking water were less virulent for mice than either *Klebsiella* from any environment of *E. coli* of clinical origins. Oral ingestion by mice of *Klebsiella* in drinking water indicated a dramatic increase in intestinal persistance when antibiotics were also administered. Recovery of viable *Klebsiella* in mouse feces was detected when as little as 10 *Klebsiella*/100ml was present in water used for drinking by these animals.

Based on the findings of a field survey of redwood reservoir water supply systems, design modifications were made to control coliform contamination. Several interrelated factors were found which affect water quality. Coliforms are not found when an adequate (0.3-0.4 ppm) free chlorine residual is maintained in the reservoir along with short retention times (less than 7 days) and a design which promotes adequate water circulation in the reservoir. The major factor influencing water quality is the chlorine demand of the wooden reservoir. It is clearly not adequate to supply incoming reservoir water with 0.3 ppm chlorine unless retention times are very short (less than 1 day) and the reservoir has been in use for over 1 year. Under no circumstances should drinking water stored in redwood be chlorinated only after leaving the reservoir. Two years of studies with an experimental 3,785 liter (1,000 gal) redwood reservoir demonstrated that coliform-free water was consistantly obtained when these design modifications were followed.

It was discovered that *Klebsielleae* are associated with the redwood lumber used to construct the reservoirs. These coliforms are found within the wood structure in numbers ranging from 10^2 to 10^6 per ml of expressed liquid. A variety of laboratory experiments demonstrated that the water extractable cyclitols comprising 4.5% redwood dry weight, provide the nutritional basis which uniquely support the growth of *Klebsielleae* in the

redwood environment. These coliforms can multiply within redwood reservoir water which leaches out cyclitols from the staves.

This report was submitted in fulfillment of Grant No. R804456 by Oregon State University under the sponsorship of the U. S. Environmental Protection Agency. This report covers the period July 20, 1976, to July 19, 1978, and work was completed as of September 1, 1978.

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SECTION 1

INTRODUCTION

The quality of drinking water in the United States has received much attention in the last several years due to the research efforts facilitated by passage of the Safe Drinking Water Act (P.L. 93-523). This Act has also resulted in an increase of the general awareness of the public to health problems associated with the consumption of inadequately treated or faulty finished drinking water supplies.

Adverse publicity in public media has brought drinking water problems into regional focus in the Pacific Northwest due to recent waterborne disease outbreaks at a ski resort in Montana, in a community in Washington State, and a public recreation area in Oregon. In addition to the personal discomforts associated with gastroenteritis, the economic impact of law suits, loss of tourism and loss of work time cost untold millions of dollars. Such waterborne disease outbreaks, are of course, not unique to the Northwest since over 54,000 individuals were reported ill from other outbreaks in the United States over the period of 1961-1973 (1). Some E.P.A. officials believe that 90% of the actual outbreaks go unreported and that numerous other illnesses are associated with the consumption of contaminated water.

The major goal of the S.D.W.A. is to assure that the public has consistantly safe drinking water. To achieve this goal, there must be routine and adequate water quality monitoring. In the summer of 1974 a coliform contamination problem occurred in a rural drinking water supply during the routine monitoring efforts of the State of Oregon Public Health Department. Upon investigation, the origin of contamination was traced to a 189,250 liter (50,000 gal) water storage reservoir constructed of redwood (Fig. 1). The coliforms were isolated and identified as Klebsiella pneumoniae and Enterobacter species. No Escherichia coli were found.

Further investigations indicated there was no obvious evidence of water contamination from insects, rodents, or birds, and the ground water sources (3 wells) were all pumping coliform-free water. Field surveys of similar water systems demonstrated that this coliform contamination in finished drinking water stored in redwood reservoirs was not an isolated situation. Coliforms were isolated from 9 of 10 private drinking water systems and from 11 of 23 water systems in state and federal parks, and in various other communities. It was not uncommon for total coliform counts to range from 5-200/100 ml of drinking water as tested by membrane filtration techniques.

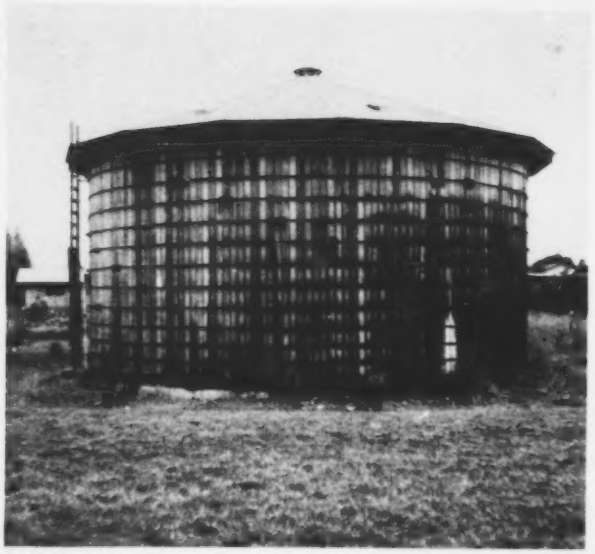


Figure 1. 189,250 liter (50,000 gal) redwood reservoir used to store finished drinking water.

Inspection of the interiors of several redwood reservoirs illustrated that on some occasions some staves were coated with a layer of slime (Figs. 2 and 3). The slime can be 0.5 cm in thickness and contains bacteria, including coliforms, embedded in mycelial states of fungal growth.

Redwood water reservoirs are widely distributed throughout the Western United States, and are also used in Canada, South America, and the Pacific Islands. They are used to store the finished drinking water needs for recreation areas, mobile home parks, motels, communities, and cities in these locations.

The purposes of this study were to determine the origin(s) and cause(s) of coliform contamination and attempt design modifications so that current as well as newly constructed reservoirs would serve potable water. The study was divided into three general phases. One phase involved an extensive characterization of the coliform Klebsiella, comparing properties of isolates from drinking water supplies with those that are derived from clinical disease in man and animals. The second phase involved the testing of design modifications in a 3,785 liter (1,000 gal) experimental reservoir, constructed for research purposes, to determine conditions which lead to coliform contamination and hopefully to their elimination. In the final phase, studies led to the demonstration of possible coliform origins and to an elucidation of the nutritional basis which facilitates the selective growth of Klebsiella and Enterobacter, the coliforms most frequently encountered in these drinking water supplies.

Figure 2 (top). Reservoir interior illustrating slime on some staves.

Figure 3 (bottom). Slime from interior ladder on concrete floor or reservoir.

SECTION 2

MATERIALS AND METHODS

BACTERIAL CULTURES ASSAYED FOR ANTIBIOTIC RESISTANCE

Klebsiella cultures received or isolated for this study were ornithine decarboxylase negative, lysine decarboxylase positive, lactose positive, citrate positive, non-motile gram negative fermentative rods. Testing of biochemical reactions and identification procedures used are those of Edwards and Ewing (2). Cultures were isolated by this laboratory from various finished drinking water sources, vegetables, redwood, sawdust, woodchips and potato processing effluents. Others were received from the Pennsylvania State University, University of Missouri, Michigan State University, University of Guelph Ontario, Canada, University of Texas (Houston), University of Oregon School of Medicine, Medical University of South Carolina, St. Lukes Hospital (Duluth, Minn.), Mercy Hospital (Pittsburgh, Pa.), B.C. Research (Vancouver, Can.), the Environmental Protection Agency at Kingston, Rhode Island, and the American Type Culture Collection.

Those cultures listed as Bovine Area were isolated from a variety of areas occupied by cows, including teat ends. It is entirely possible that those isolates from bedding and stall water may be recent contaminants from animals that were frequently fed antibiotics both for prophylaxis and treatment.

E. coli strain W3110 (Nal^r, F^-) was obtained from the laboratory of Dr. Lyle R. Brown (Oregon State University).

Antibiotic Sensitivity Testing

Sensitivities were determined by the high concentration disk diffusion technique as described by Bauer, et al. (3). The following antibiotic disks were used: penicillin G (10 U, Pen), streptomycin (10 mcg, Str), chloramphenicol (30 mcg, Chl), nitrofuradantoin (300 mcg, Nitro), kanamycin (30 mcg, Kn), tetracycline (30 mcg, Tc), neomycin (30 mgc, Nm), ampicillin (10 mcg, Am), erythromycin (15 mcg, Ery), novobiocin (30 mcg, Nov), nalidixic acid (30 mcg, Nal), gentamicin (10 mcg, Gent), polymyxin B (300 U, Poly), furaltadone [30 mcg, (Valsyn, Val)] and nitrofurazone (100 mcg, Furacin). Furaltadone discs were obtained from BBL, nitrofurazone from Pfizer and the remainder were purchased from Difco. Bacto Mueller-Hinton (Difco) plates were inoculated and after 18 to 20 h incubation at 37°C, zone diameters were measured. Isolates were assigned as resistant or not

resistant according to the standards in the Manual of Clinical Microbiology (4).

Transfer of Multiple Resistance

Conjugal transfer of multiple antibiotic resistance was attempted for 31 Klebsiella isolates using E. coli strain W3110 as recipient and for 5 isolates using Klebsiella as recipients. All chosen presumptive donors were resistant to two or more antibiotics. Since all Klebsiella strains are resistant to penicillin in standard doses, this antibiotic was excluded from consideration as were novobiocin and erythromycin because they are used primarily in the treatment of infections caused by gram positive organisms.

Spontaneous nalidixic acid resistant mutants (40 ug/ml) of Klebsiella isolates with low minimum inhibitory concentrations (MICs) for Str, Tc, Chl, Val, Nitro, Kn and/or Nm were selected for use as presumptive recipients.

MICs for antibiotics were determined for donors on MacConkey agar where resistance to two or more antibiotics was detected. The recipient organisms were likewise tested and concentrations between the MICs of donor and recipient were used in the selective media. Preparation of donor and recipient cell suspensions followed standard procedures (5). After incubation, appropriate dilutions of the mixtures were then plated on selective media containing nalidixic acid and combinations of antibiotics to which the donors were resistant. Aliquots were also plated on MacConkey agar supplemented with nalidixic acid only to determine the total number of recipients. Frequency of transfer of a given resistance pattern was taken as total number of transconjugants/total number of recipients.

COMPARATIVE PATHOGENICITY EXPERIMENTS

Bacterial Cultures

The origins and identification of most Klebsiella isolates have been previously described (6). Klebsiella isolates were of the following origins: 19 mastitis, 18 human clinical, 18 bovine-related areas and 42 from other environmental sources. In addition, E. coli were obtained from drinking water (6 isolates) and from human infections (6 isolates). Additional bovine mastitis and other bovine-related Klebsiella (6) were obtained from the University of Guelph, Guelph, Ontario, Canada. Bovine-related isolates were divided into two groups: bovine-area (from unused and used bedding and drinking water in stall areas) and bovine-associated (from mouth, udder, and anal areas). Klebsiella isolates were divided into two species, K. pneumoniae and K. oxytoca, according to the scheme of Naemura and Seidler (7).

LD_{50} Assay

Test cultures were shaker-grown for 18 to 20 hr at 37°C in nutrient broth (Difco, Detroit, Mich.) with 1% added glucose. Three-fold dilutions

for inoculation were made in steam-sterilized 5% hog gastric mucin (Sigma, St. Louis, Mo.). Five 17-20 gm male Swiss-Webster mice were injected i.p. with 1.0 ml of each dilution; five to six dilutions were injected per culture. Virulence was determined by the numbers of mice dying within 48 h after inoculation and with recoverable test organisms from heart and liver sections at autopsy.

LD_{50} Computation

Cell numbers for each inoculated dilution were transformed into natural log values. Each LD_{50} was determined using a maximum likelihood computer program. This type of logit analysis is similar in principle to the use of probit transformations (8).

Statistical Tests

Geometric mean LD_{50} values were compared using F tests and one- and two-tailed t tests, at an $\alpha = .05$ significance level (9). All calculations were made using natural log LD_{50} values.

FECAL COLIFORM RESPONSE

Determination of Fecal Coliform (FC) Response

For membrane filtration (MF) FC tests, 24-hr shake cultures grown at 35°C were diluted to approximately 10 cells/ml. Duplicate 3-ml volumes were filtered using 0.45 µm-pore-size membrane filters (GN-6, Gelman, Ann Arbor, Mich.) with cultivation on m-FC medium (Difco, Detroit, Mich.) for 24 hr at 44.5 (10). Colony number and color were recorded on each plate after incubation.

All isolates were verified by subculturing a blue colony to phenol red lactose broth (Difco) for 24 hr at 35°C with subsequent transfer to EC broth at 44.5°C. Tubes producing gas were considered confirmed FC. Isolates having blue-green or light blue-gray colonies were not considered MF FC positive. These colony types had a low percent confirmation both in this and other reported surveys (11).

For MPN FC tests, cultures were first grown in phenol red lactose broth at 35°C. After 24 hr, cultures were transferred to EC broth and incubated for 24 h at 44.5°C. Tubes with growth and gas production were considered FC positive.

pH of Cultures in m-FC Broth at 44.5°C

Selected isolates of *K. pneumoniae* and *E. coli* were incubated in tubes containing 7 ml of m-FC broth (Difco) containing 0.01% rosolic acid (Difco) for 24 hr at 44.5°C. Direct pH measurements of the broth were made with a combination triple-purpose electrode (no. 476022, Corning Glassware, Corning, N.Y.). Broth color was recorded immediately upon removal from the incubator.

Statistical Testing of Results

Results from FC tests for K. pneumoniae and E. coli isolates from various sources were analyzed for statistical differences using chi-square 2 x 2 contingency tables (9). Differences were considered significant if P values were less than 0.05.

KLEBSIELLA PERSISTANCE IN THE MOUSE INTESTINAL TRACT

Test Organisms

Six Klebsiella cultures were used throughout these experiments, representing pathogenic and environmental origins (three each). The K. oxytoca, FC-positive (FC+) K. pneumoniae, and the FC-negative (FC-) K. pneumoniae used were represented by two cultures each. The habitat of origin of the Klebsiella cultures were: two human clinical and one from bovine mastitis, fresh vegetable, finished drinking water, and redwood sawdust chips. The isolation and biochemical characteristics of these cultures have been described (6).

All cultures were made resistant to 40 µg/ml nalidixic Acid (Nal) and 500 µg/ml streptomycin (Str) (both obtained from Sigma, St. Louis, Mo.) to facilitate culture recovery from fresh mouse feces. Antibiotic (AB) resistance was chromosomal and transfer via plasmids to other intestinal bacteria was not detected. Cultures were stored on nutrient agar (Difco, Detroit, Mich.) slants with Nal-Str until used in experiments.

Ingestion of Klebsiella in Drinking Water

Cultures were shaker-grown in brain heart infusion (BHI) broth (Difco) with Nal-Str for 18-20 hr at 37°C and diluted to 10^5, 10^3, 10^1, or 10^{-1} cells/ml in sterile distilled water. 100 ml of each dilution was given to each of eight mice as a sole source of drinking water (DW). This DW was changed every 24 hr. Culture survival or regrowth was determined by plate counts on Nal-Str-containing MacConkey agar (Difco). Culture regrowth in sterile DW was found to be limited, i.e., no more than a half log increase over original numbers within 24 hr. Mice were observed to ingest a maximum of four ml of water in a 24 hr period. Over this period, mice given an initial level of 10^0 cells/ml DW, for example, would be ingesting three to eight cells of Nal-Str resistant Klebsiella. After six days of continuous exposure to Klebsiella in DW, non-sterile tap water was placed into cleaned water bottles for the remainder for the experiments.

Test animals were individually housed in sterile, plastic cages lined with kiln-dried wood shavings. Food pellets were supplied ad libitum. Fecal pellets were collected immediately prior to transfering mice to fresh, sterile cages.

Culture Recovery from Feces

One-tenth g of fresh fecal pellets were collected from each test mouse, using flame-sterilized forceps, on days 2, 4, 6, 7, 14, 21, and 28 after ex-

periments were begun. Fecal pellets of mice ingesting 10^{-1} Klebsiella/ml DW were collected every day for up to nine days. Pellets were emulsified in sterile tubes containing 0.9 ml of BHI broth with Nal-Str, providing a 10^{-1} dilution of the fecal material. Subsequent dilutions were made in the same broth using either a 0.01 ml capacity inoculating loop or a 0.1 ml pipet.

After 18 to 20 hr incubation at 37°C, dilution tubes showing growth were streaked onto Nal-Str-containing MacConkey agar. Plates were incubated at 37°C for 22 to 24 hr. Dilutions producing red or red-centered colonies on this selective agar were considered presumptive for presence of the orally-ingested Klebsiella. Standard biochemical tests confirmed the identification (2). No organisms other than the Nal-Str resistant Klebsiella grew on this medium.

The number of Nal-Str resistant Klebsiella/g of feces was calculated as one over the highest dilution having colonies on the MacConkey agar plates. The detection limit for this method was one Nal-Str resistant cell/0.1 g feces. This was experimentally determined by seeding mouse feces with known amounts of Klebsiella.

Effect of Oral Antibiotics (AB) on Mice Ingesting Klebsiella

In these experiments, mouse DW contained 40 µg/ml Nal and 500 µg/ml Str at all times. This AB water was supplied to the mice 48 hr prior to addition of Klebsiella to the same drinking water. Mice were then given fresh Nal-Str with Klebsiella water every 24 hr. After six days, cultures were removed from DW. Fresh AB water alone was changed every 48 hr for the remainder of the experiments. Partial decontamination of the intestinal tract was achieved by this prior oral AB exposure. Total coliforms were reduced by 99% (10^6 to 10^4/g feces using MacConkey agar) and facultatively anaerobic bacteria by 99.9% [10^9 to 10^6/g feces using standard plate count agar (Difco) with overlay]. Klebsiella densities ranged from 10^{-1} to 10^3 cells/ml drinking water in these experiments. Techniques for fecal pellet collection and culture enumeration were the same as described above.

Effect of Cyclophosphamide (CP) on Mice Ingesting Klebsiella

Test mice were injected with 200 mg/kg CP (Cytoxan, Meade Johnson Lab., Evansville, Ind.) three days after Klebsiella were first added to DW. Mice ingesting 10^1 to 10^5 Klebsiella/ml DW without AB (control mice) and 10^1 to 10^3 Klebsiella/ml DW with AB were tested in these experiments. Techniques for fecal pellet collection and culture enumeration were the same as described above.

The effects of comparable doses of CP on white blood cell counts and other immune-response system functions have been previously reported (13).

EXPERIMENTS ON A 3,785 LITER (1,000 GAL) EXPERIMENTAL REDWOOD RESERVOIR

A 3,785 liter (1,000 gal) redwood tank was constructed for use in this investigation by National Tank and Pipe Co., Portland, Ore. It measured 1.80 m (71 in.) in height with an interior radius of .84 m (33 in.). The 42 staves of the tank each measured 6.51 cm (2 1/16 in.) by 13.65 cm (5 3/8 in.). A hinged plywood lid covered the top. Incoming water, from municipal city lines, entered the tank at the top and was sprayed evenly to all sides. A float system controlled the level of the water and drainage was through a valve at the bottom of the tank (Fig. 9).

Various retention times for tank water were achieved with a motor timer which controlled a solenoid valve. The timer monitored both the length of time between drainings (Timer #1) and the length of time the drain was open (Timer #2).

Chlorine concentrations in the incoming water were regulated by a metering pump on 303 liter (80 gal.) holding tank. The stock chlorine solution was prepared by adding 3.79 liters (1 gal.) of commercial bleach to 53 liters (14 gal.) of tap water (1:15 dilution). A dial on the pump was adjusted to provide appropriate chlorine concentrations for water entering the tank.

Retention Times

It was found that water drained from the tank at the rate of 11.7 liters (3.1 gal.) per minute. The timers were adjusted to provide retention times based on this drain rate.

Preliminary Adjustments and Decontamination of Tank Interior

The manufacturer's recommendations were followed. In order to leach tannins from the wood, which affect odor, color, and taste of the water, 2.27 kg (5 lb.) of soda ash was added to a full tank and held for 24 hr. After drainage, enough stock chlorine solution was added to bring the chlorine concentration in the full tank to 200 ppm. Granular dry chlorine was used in preparing the stock solution. The tank was put into service in October 1975; however, in November 1975 it was necessary to perform a similar soda ash treatment of 7 days duration. This second treatment greatly improved the taste, odor, and color of the water.

Preliminary studies with the tank at 20 percent capacity were then carried out between the first month of operation and the third month to determine the amount of chlorine necessary to achieve an adequate free chlorine residual (0.2 to 0.4 ppm) in the drain (outlet) water. The effect of lengthened retention time on residual chlorine was also examined. During this period, bacteriological examinations of the water were performed regularly. No coliforms were detected during this period; total bacterial counts averaged 350 per ml and never exceeded 2000 per ml.

During the third month, the experimental tank was filled to capacity and set on a 1 day retention time. With this schedule, approximately one-sixth of the tank water was drained every 4 hrs. Capacity was maintained at all

times by a float system. Retention times were later increased to two, four, eight and finally twelve days. During the sixteenth month, the chlorine amendments were terminated in order to observe the effects of lowered chlorine residuals on the bacteriological quality of tank water; water entering the tank then carried a free residual of 0.5 ppm, due to the chlorination of the municipal water.

Daily observations of physical parameters of tank water

Free chlorine residuals and total chlorine residuals in the drain water and inlet water were determined by the N,N-diethyl-para-phenylene-diamine (DPD) method. Optical density readings and pH were also determined for water samples.

Bacteriological examination of tank water and staves

Water leaving the tank via the drain at the bottom was periodically examined for total coliforms and total bacterial counts, using standard procedures (10). Total coliforms were enumerated by the membrane filtration technique on m-Endo agar LES. Sodium thiosulfate was added to all samples to remove any free chlorine in the water. Varying amounts of drain water (usually 100 ml or 1000 ml) were tested. On eight occasions, the water level was lowered and the interior surfaces of the tank were scraped with sterile glass microscope slides. Surface material was dispersed and tested for total coliforms by the spread plate technique on m-Endo agar LES and Endo agar, and with phenol red lactose broth. In addition, some non-coliform bacteria were isolated for identification.

Identification of Isolates from Staves

Media preparation and procedures used for the identification of coliform organisms were those recommended by Edwards and Ewing (2). Final identifications were made using the API 20E System. Isolates obtained from plate count agar were identified using 9-digit profiles of the API 20E System, or by procedures outlined in the eighth edition of Bergey's Manual of Determinative Bacteriology (13,14).

Nutrient Potential of Redwood Tank Water

Samples of water from the 3,785 liter (1,000 gal) experimental tank were collected at various times during the investigation and stored at -17°C. The samples were thawed and filter sterilized using membrane filters. They were inoculated with low cell densities (less than 100 cells/ml) of an 18 hr culture of Klebsiella pneumoniae and incubated with shaking at 35°C. Plate counts were performed at 48 hr and 96 hr. All glassware was acid washed to remove any extraneous nutrient sources. Sodium thiosulfate was used to remove chlorine when necessary.

COLIFORM BACTERIA WITHIN WOOD

Wood Samples

Samples of fresh and aged redwood were obtained from National Tank and Pipe Company, Portland, Ore., and Simpson Lumber Company, Arcata, Cal. Samples of other wood were obtained from mills in Oregon.

Liquid Extraction from Wood

All types of wood had outer surfaces removed using a power circular saw. The sawblade and cutting table were surface sterilized with 95% ethanol. Swabs of these surfaces were routinely streaked onto m-Endo agar LES (Difco, Detroit, Mich.) to ensure absence of external contamination. The wood samples were then cut into blocks approximately 131 cm^3 (8 in^3) in size. Moisture content of the wood was determined by measuring sample weight both before and after drying at 60°C for 7 days.

Liquid was extracted from the wood blocks using a Carver Laboratory hydraulic press (Fred S. Carver, Inc., Summit, N.J.) operated at up to 844 kg/cm^2 (12,000 psi). Blocks were placed on sterile aluminum foil with upturned edges for liquid collection. During pressing, the blocks were flooded with 2 ml of sterile 1:10 w/v polyvinyl pyrrolidone (PVP) to neutralize any released phenolic compounds. Expressed liquid was transferred to sterile tubes for bacterial testing.

Bacterial Enumeration

Dilutions of liquid expressed from wood were made in sterile, 0.01 M Tris (pH 7.5). One-tenth ml of appropriate 10-fold dilutions were spread onto media for viable cell counts. Total bacterial counts were made on nutrient agar (Difco), with incubation at 30°C for 48 hr. Standard plate count procedures were not used since total bacterial counts are lowered by brief exposure to the elevated temperature of molten agar (41). Also, the possible loss of a selected portion of the bacterial population was of concern. The procedures and diluent used in determining heterotrophic counts from wood were at least as sensitive if not better than those of Standard Methods (see Appendix). Total coliform counts were made using m-Endo agar LES, with incubation at 35°C for 24 hr. In one experiment with redwood collected directly from a California mill, both total bacteria and total coliform counts were made at 37°C to enhance selection of Klebsiella (15). Using non-redwood samples, most probable number (MPN) techniques were also used to obtain confirmed total coliform counts (10). Fecal coliform counts were made using m-FC agar (Difco), with incubation at 44.5°C for 24 hr in a water jacketed incubator (NAPCO #3221).

Cut Surface Contact Impressions

Outside surfaces of wood samples were removed as described above and impressions were made by bringing a freshly exposed surface into contact with m-Endo agar LES. With two samples of fresh redwood, six to nine successive

.6 cm (1/4 in.) sections were cut through each block, after outer surface removal. An impression of each successively exposed surface was made on m-Endo agar LES. All plates were examined for growth and green-sheened colonies after 24 and 48 hr incubation at 35°C. Total coliform counts were based only on the number of typical colonies appearing after 24 hr.

Coliform Identification

Confirmed coliform isolates from all wood experiments were tested biochemically for identification. A green-sheened colony from m-Endo agar LES was considered to be a confirmed coliform if it produced gas in lactose broth, within 24 hrs at 35°C. Presumptive speciation was made using Triple Sugar Iron Agar, urea, and Simmons' citrate agars, lysine and ornithine decarboxylase broths, and motility tests. Further identification and characterization was made using standard biochemical tests (2) and the API 20E system (Analytab Products, Inc., Plainview, NY). *Klebsiella* isolates were speciated according to Naemura *et al*. (L.G. Naemura, S.T. Bagley, and R.J. Seidler. Abstr. Annu. Meet. Am. Soc. Microbiol. 1977. I 149, p. 179) and (7).

Electron Photomicrographs of Wood

Samples 0.2 to 0.5 cm^3 were cut from redwood blocks, processed through solutions of ethanol and trichlorotrifluroethane in increasing concentrations, and dried by the critical point method of Cohen, et al. (16). After drying, the cubes were split parallel to the longitudinal axis of the wood fibers to expose fresh internal surfaces. These pieces were fastened to aluminum planchets and coated with approximately 200 A° of 60:40 gold/palladium. Samples were observed at 15 Kv in an ISI Mini-SEM MSM-2 scanning electron microscope and images recorded on Type 55 Polaroid film.

NUTRITIONAL EXPERIMENTS

Aqueous Redwood Extracts

Aqueous extracts of redwood sawdust used in these experiments were prepared in the following manner: 1.67g or 4g of redwood heartwood sawdust was placed into 1 liter of sterile double distilled water and allowed to stand at room temperature for 4 days. At this time, sterile 0.1% sodium hydroxide was used to neutralize the extract. The extract was sterilized by membrane filtration (0.22 um) and pipetted into sterile flasks for the growth experiments. (Preliminary studies showed that the aqueous extract prepared from 1.67g/liter sawdust resulted in optimum growth of inoculated organisms capable of utilizing cyclitols). The 4g/liter sawdust extract was used only for quantitating cyclitols in redwood sawdust extracts. Non-sterile extracts were not membrane filtered.

Preparation of Samples for Gas Chromatography

In all cases 0.5 ml samples of redwood extract or tank water were processed. Quebrachitol (a gift from Dr. Laurens Anderson, Dept. Biochemistry,

University of Wisconsin) a cyclitol not present in redwood, was used as an internal quantitation standard. 0.05 ml of a 1 mg/ml aqueous solution of quebrachitol was added to all samples before dry heat evaporation. In the case of the more concentrated sawdust extract (4g/liter) it was necessary to remove contamination from phenolic compounds. This was achieved by lead acetate precipitation (17).

Dry samples were silylated with .2 ml of Tri Sil Z (Pierce Chemical Co., Rockford, Ill.) for 3 hr at room temperature and 2 µl was injected into the gas chromatograph.

A Hewlett-Packard Model 5710A gas chromatograph with a flame-ionization detector was used in these experiments. This instrument was equipped with a 6 ft. 3% OV-17 stationary phase on a Gas Chrom Q support (100-12 mesh) for analysis of redwood extracts. The oven temperature used was 160 C, with a carrier gas (helium) flow of 20ml/min. Injection port temperature was 260°C; detector temperature was 300°C. Peaks were recorded with a Hewlett-Packard Model 7123A strip chart recorder. The FID control was set with a range of 10 and attenuation of 2 for all analyses.

Quantitative analyses were determined by first preparing a tracing on uniform thickness bond paper. The peaks were cut out and weighed on a Metler analytical balance and the mass compared to that of the internal standard.

Preparation of Bacterial Cultures for Nutritional Experiments

Cultures were grown overnight in Difco penassay broth at 28°C. Cultures were diluted to 10^{-6} through sterile 0.01M Tris buffer, pH 7.5 and inoculated into the sawdust extract to achieve an initial density of about 10^2 cells/ml. Tris buffer diluent provided viable counts indistinguishable from those obtained when Standard Methods buffer was used (see Appendix). Appropriate media controls were used to assess growth in distilled water or basal salts medium without a carbon source. Cultures examined for utilization of cyclitols in a defined medium were propagated in basal salts medium in the presence of a growth limiting amount of glucose (0.02%).

Basal Salts Medium

A defined medium was used to test for cyclitol utilization as a sole source of carbon and energy. The medium contains the following ingredients in g/l: Tris buffer to pH7, 10g; NH_4Cl, 2g; KCl, 2g; KH_2PO_4, 1g; Na_2SO_4, 0.1g (solution A). Solution B contains in g/100 ml: $CaCl_2 \cdot 2H_2O$, 0.05g; $MgCl_2$, 0.02g. To prepare 1 liter of medium, combine 900 ml sterile solution A, 10 ml solution B, and add 100 ml of the test carbon source to achieve 0.2% of the latter.

SECTION 3

RESULTS AND DISCUSSION

BIOCHMEMICAL PROPERTIES OF THE KLEBSIELLA

Table 1 summarizes the biochemical test responses of redwood associated Klebsiella and those published by Edwards and Ewing (2). All isolates conform to the current taxonomic criteria for K. pneumoniae (2,4). The only significant deviation in incidence of positive reactions was found in the tests for indole production and urease activity. A significantly greater incidence of indole production was observed among the clinical (human-derived) and redwood associated isolates compared to the frequency compiled by Edwards and Ewing. The environmental group also had a smaller number of urease-positive isolates.

The percent variability in urease reaction between sources is similar to data reported by Henriksen (18) in which 96% of his pathogenic and fecal Klebsiella strains were urease positive and 69% of the water strains were positive. Positive urease activity has been suggested by Buttiaux (19) and Mossel (20) as indicative of Klebsiella strains of fecal origin. All FC-positive isolates in this study were urease positive. However, all but one of the FC-negative pathogenic strains and 68% of the environmental FC-negative strains were also urease positive. Thus, in using the current concept and techniques for FC detection, urease activity alone would not be sufficient to indicate that a Klebsiella isolate is of fecal origin.

The indole-positive isolates, which appear otherwise to be K. pneumoniae by routine biochemical tests, resemble the "oxytoca" group as proposed by Lautrop (21), Stenzel et al., (22), and Von Reisen (23). Others have illustrated that indole-producing Klebsiella are present in significantly higher frequencies in human feces and clinical material than might be indicated from the compilations of Edwards and Ewing. Thus, regardless of indole and urease responses, all redwood associated isolates obtained in the present studies are indistinguishable from what others have also referred to as Klebsiella and such isolates are described from human/animal infections and from human fecal material.

The field surveys on the bacteriological quality of finished drinking water emanating from redwood tanks illustrated that Klebsiella is usually not accompanied by the presence of E. coli (24). Because of the ubiquity of Klebsiella in other botanical environments apparently free from obvious fecal pollution, the immediate health importance of Klebsiella has been underestimated (11,25). Identification of Klebsiella has also been confused with

TABLE 1. BIOCHEMICAL REACTIONS OF REDWOOD ASSOCIATED KLEBSIELLA ISOLATES COMPARED TO DATA GIVEN BY EDWARDS AND EWING (3)

Test of substrate	Redwood Associated[a] (% +)	Clinical (Edwards and Ewing) Sign[b]	Clinical (Edwards and Ewing) % +
Indole	33	-	6.0
Methyl red	14	- or +	13.3
Voges Proskauer	92	+	91.1
Simmons' citrate	100	+	97.7
H_2S (TSI)	18	-	0
Urease	75	+	94.5
KCN	71	+	97.7
Motility	0	-	0
Gelatin	5	-	3.3
Lysine decarboxylase	100	+	97.2
Arginine dihydrolase	0	-	0
Ornithine decarboxylase	0	-	0
Phenylalanine deaminase	0	-	0
Malonate	80	+	92.5
Glucose: Acid	100	+	97.0
Gas	94	+	96.5
Lactose: Acid	100	+	98.2
Gas	100	+	98.0
Sucrose	100	+	98.9
Mannitol	100	+	100.0
Dulcitol	69	-or +	31.5
Inositol: Acid	100	+	97.9
Gas	80	+	91.9
Arabinose	100	+	99.9
Rhamnose	83	+	99.3
Glycerol	100	+	93.0

[a]Isolates obtained from field and laboratory redwood reservoirs and from redwood sawdust chips.

[b]The sign indicates: +, 90% or more positive in 1 or 2 days; -, 90% or more negative; - or +, majority negative.

that of Enterobacter (Aerobacter), and Klebsiella has been grouped as a total coliform. However, the fecal coliform (FC) elevated temperature test is considered a most relevant test for examining the fecal origin of coliform bacteria (26). Klebsiella is carried in the intestinal tract of some 30 to 40% of humans and animals and some environmentally-derived Klebsiella isolates have given a positive response in this test (11,19,27-30). The applicability of the FC test response in the present studies seemed relevant except for some important missing information. That is, no one had examined the incidence of the FC test response among known pathogenic isolates of Klebsiella. Tables 2-4 summarize results of a comparative analysis on the FC test responses of 191 Klebsiella of pathogenic and environmental sources.

Using standard MPN techniques (10), approximately 85% of the human and animal pathogenic isolates were FC positive as judged by gas production from lactose when incubated at 44.5°C compared to approximately 17% of the environmentally-derived isolates. A breakdown of the FC responses of environmental Klebsiella is provided in Table 3.

Isolates obtained from small experimental 65 liter redwood tanks (24), from redwood sawdust and wood chips, from finished public drinking water supplies, and from fresh vegetables account for most of the FC-positive results using both MPN and membrane filter (MF) technique. The high percent confirmation of MF FC-positive Klebsiella from all sources indicates they are true fecal coliforms and conform to the operational definition of a coliform of fecal origin (10,26). Although the original source of the FC-positive redwood associated Klebsiella has not and cannot be determined with certainty (origins from insects, birds, animals, or humans) their presence is made more significant in view of the 85% FC-positive incidence among Klebsiella isolates of known clinical origins and strongly supports their sanitary significance.

The presence of high Klebsiella numbers in botanical environments in general results from their regrowth (31); (see later sections). This regrowth further complicates any interpretation of their sanitary significance in drinking water supplies emanating from redwood reservoirs. Thus, the occurrence of FC-positive Klebsiella is indicative of fecal pollution at some point in time which may have been recent or much earlier. The presence of specific cell densities of Klebsiella in the absence of E. coli does not imply the presence of other pathogenic enteric bacteria. The deterioration of environmental quality is based on the opportunistic pathogenic nature of Klebsiella per se and a recognition of the nature by which exposure and subsequent clinical manifestation of infection are generally separated in time.

Studies have supported the contention that indole producing Klebsiella pneumoniae should be considered as a separate taxon (K. oxytoca or Oxytocum) and not, as in the present context, as FC negative K. pneumoniae (7,21-23). Such taxonomic considerations, which the data support, have a relevant impact on the FC surveys. For example, the incidence of FC positive Klebsiella among the known pathogenic isolates changes significantly since all but one of the FC negative cultures was indole producing.

TABLE 2. MPN AND MF FC-POSITIVE KLEBSIELLA CULTURES

Origin	No. of isolates	Positive FC response (%)		% Confirmation[a]
		MPN	MF	
Klebsiella pneumoniae				
Human clinical	46	82.6	71.7	93.6
Mastitis	22	86.3	86.3	100.0
ATCC	3	66.7	66.7	100.0
Environmental	120	16.7	15.8	89.5

[a]Numbers indicate the percentage of MF FC-positive isolates confirmed in EC broth.

TABLE 3. FC RESPONSE OF ENVIRONMENTAL KLEBSIELLA ISOLATES

Origin	No. of isolates	Positive FC response (%)		% Confirmation[a]
		MPN	MF	
Redwood lab tanks	11	27.3	27.3	100.0[a]
Public drinking water	43	16.3	16.3	85.7
Private drinking water	21	0.0	4.8	0.0
Redwood sawdust and chips	15	33.3	33.3	100.0
Vegetables	19	21.1	15.8	100.0
Industrial effluents[b]	11	9.1	9.1	100.0

[a]See footnote, a, Table 2.

[b]Includes pulp and paper mill (five), textile mill (four), and potato processing effluent (two).

Thus, nearly 100% of the K. pneumoniae sensu stricto of clinical origins are fecal coliforms. One-third of the 120 environmentally-derived Klebsiella were indole producing. Thus, the actual incidence of FC positive K. pneumoniae from the environment was at least 19 of 80 or some 24%. The sanitary significance of the oxytoca group should not be discounted either, even if FC negative, since 34% of Klebsiella fecal isolates and 17% of human clinical isolates (15% in the present survey) have been reported as indole-producing.

In the present survey, Klebseilla fecal coliform positive colony color and morphology ranged from typical flat and dark blue with or without precipitated bile, to raised, mucoid, dark blue, to raised with blue centers and cream-colored edges. Also, some 40% of the fecal coliform positive cultures exhibited flat, dark blue colonies with some orange coloration. Because of this diversity in reaction on mFC agar, experiments were conducted to determine whether pH in mFC broth (with rosalic acid) could be used to correlate with the patterns of FC response (MPN and MF) and observed colony morphology. The results from 42 Klebiella able to grow at 44.5°C (aerogenic or anaerogenic) and 12 FC positive E. coli are illustrated in Table 4.

A distinct response pattern was indeed recorded correlating final pH, colony morphology, and responses with MF and MPN techniques. Thus, the Klebsiella and E. coli which were MF+, MPN± had pH < 5.3; MF(-), MPN+ isolates had a pH range of 5.3 to 5.8, while MF(-), MPN(-) isolates had a pH greater than 5.8.

Klebsiella isolates with typical dark blue colonies on mFC agar or blue-orange colonies produced the lowest pH (<5.3). Klebsiella cultures which were MF(-), MPN+ had blue-centered colonies with cream colored edges or were light blue-gray or blue-green in appearance. MF(-), MPN(-) cultures produced pink or cream colored colonies on mFC agar.

ANTIBIOTIC RESISTANCE OF THE KLEBSIELLA

In order to further characterize Klebsiella from finished drinking water, other related environments, and from clinical infections, some 183 isolates were tested for resistance to 11 antibiotics. Some isolates found to be resistant to 2 or more antibacterials ("multiple resistant" in this study) were also tested for their ability to transfer resistance to E. coli and to other Klebsiella isolates. Results of these experiments are presented in Tables 5 to 7.

Table 5 summarizes the percent of isolates resistant to antibiotics. Although antibiotic resistance was recorded for isolates associated with water stored in redwood reservoirs, with industrial effluents and with other botanical material, as might be expected the greatest incidence of resistant isolates was observed among the human and animal clinical specimens. The high incidence of resistance to Pen and Amp would be expected for gram-negative bacteria and this observation carries no relevant health significance.

TABLE 4. CULTURE pH IN m-FC BROTH TEST INCUBATED AT 44.5°C VERSUS FC RESPONSE

	FC response		No. of isolates	pH range (% occurring)		
	MF	MPN		<5.3	5.3-5.8	>5.8
Klebsiella pneumoniae						
Clinical	+	+	9	66.7	33.3	0.0
	+	(-)[a]	3	100.0	0.0	0.0
	(-)	+	5	0.0	100.0	0.0
	(-)	(-)	4	0.0	0.0	100.0
Mastitis	+	+	8	100.0	0.0	0.0
Environmental	+	+	6	66.7	33.3	0.0
	+	(-)	1	100.0	0.0	100.0
	(-)	+	3	0.0	100.0	0.0
	(-)	(-)	3	0.0	0.0	100.0
Escherichia coli						
Human clinical	+	+	7	71.4	28.6	0.0
Environmental	+	+	5	100.0	0.0	0.0

[a]Parenthesis indicate growth but a FC-negative reaction.

TABLE 5. ANTIBIOTIC RESISTANCE OF KLEBSIELLA ISOLATES FROM VARIOUS HABITATS

		PERCENT RESISTANT														
ORIGIN	NUMBER TESTED	PEN	STR	CHL	NITRO	KN	TC	NOV	NM	ERY	AMP	NAL	GENT	POLY	VAL	FURACIN
Mastitis	29	100	37.9	6.7	6.7	3.5	17.2	100	3.5	100	72.4	0	0	0	24.1	0
Human Medical	57	100	17.5	1.8	40.5	8.8	24.6	100	10.5	96.4	98.3	3.5	1.8	0	19.3	0
Total Medical	86	100	24.4	3.5	30.2	7.0	20.9	100	8.1	97.6	90.7	2.3	1.2	0	23.3	0
Bovine Area	23	91.3	47.8	4.4	0	0	30.4	100	0	100	52.2	0	0	0	17.4	0
Water	27	100	7.4	0	0	7.4	0	100	0	100	66.7	0	0	0	0	0
Vegetable	19	100	0	0	10.5	0	0	100	0	100	78.9	0	0	0	0	0
Pulp Mill	12	100	0	0	8.3	0	0	100	0	100	91.7	0	0	0	0	0
Other	16	100	12.5	0	0	0	12.5	100	0	100	81.3	0	0	0	18.8	0
Total Environment	97	98.9	15.5	1.0	3.1	2.1	9.3	100	0	100	72.2	0	0	0	7.2	0

Within the clinical group of cultures, those of human origins were resistant to Nitro, Kn, and Nm in substantially greater percentages than the mastitis isolates. The opposite was observed for Str. The incidence of Val resistance among the human isolates (19.3%) was very similar to that of the mastitis isolates (24.1%) even though the drug is presumably not available for human use.

Among the remainder of the isolates from various environments (excluding bovine area), resistance to Str, Nitro, Kn, Tc, and Val were recorded. Resistance to two antibiotics was recorded from four of 27 Klebsiella isolated from redwood tanks. It must be indicated that 3 of these 4 isolates came from one small experimental laboratory redwood tank originally filled with finished municipal drinking water. No coliforms were ever detected in this municipal water which carried a total chlorine residual (OTO technique) of 0.3-0.5ppm. The tank water contained no free or combined chlorine. Two of these isolates were resistant to Amp, Str while the third was resistant to Amp, Kn. The remaining Klebsiella isolate (Amp, Kn) was isolated directly from the finished drinking water stored within a redwood reservoir on a public water supply system.

Table 6 presents the drug resistance patterns of Klebsiella resistant to two or more antibiotics. It is apparent that those isolates with resistance to three or more drugs were almost exclusively from clinical origins. The two exceptions were isolates from textile mill effluents. A total of 20 different multiple-resistance patterns were found among the human medical isolates. Of the 27 "water" isolates, 22 were obtained from finished drinking water emanating from public or privately owned redwood tanks and only 1 (Amp, Kn) was resistant to 2 antibiotics. Other Klebsiella from botanical environments were found resistant to two or more antibiotics. These included 3 from redwood chips, 1 from pulp mill effluent, 2 from textile mill effluents and 2 from the surfaces of fresh vegetables.

TABLE 6. DRUG RESISTANCE PATTERNS OF MULTIPLE-RESISTANT KLEBSIELLA ISOLATES

RESISTANCE PATTERN	Mastitis (29)	Human Medical (57)	Bovine Area (23)	Water (27)	Vegetable (19)	Pulp Mill (12)	Other (16)
amp, str	3	1	2	2	-	-	-
amp, val	4	2	2	-	-	-	3

(continued)

TABLE 6. (continued)

RESISTANCE PATTERN	Mastitis (29)	Human Medical (57)	Bovine Area (23)	Water (27)	Vegetable (19)	Pulp Mill (12)	Other (16)
amp, nitro	-	11	-	-	2	1	-
amp, tc	-	3	1	-	-	-	-
amp, kn	-	1	-	2	-	-	-
str, tc	2	-	5	-	-	-	-
str, chl	1	-	-	-	-	-	-
amp, str, tc	1	-	-	-	-	-	2
amp, nitro, val	2	3	-	-	-	-	-
amp, nitro, str	1	2	-	-	-	-	-
amp, str, chl	1	-	-	-	-	-	-
amp, str, val	-	1	-	-	-	-	-
amp, tc, val	-	1	-	-	-	-	-
amp, nitro, nal	-	2	-	-	-	-	-
amp, nitro, tc	-	1	-	-	-	-	-
amp, tc, chl	-	1	-	-	-	-	-
amp, str, tc, val	1	-	1	-	-	-	-
amp, nitro, nm, val	-	1	-	-	-	-	-
amp, str, tc, nitro	-	1	-	-	-	-	-
amp, tc, nm, kn	-	1	-	-	-	-	-
amp, str, tc, nm, val	-	1	-	-	-	-	-
amp, str, tc, nitro, val	-	1	-	-	-	-	-
amp, str, tc, kn, nm,	-	1	-	-	-	-	-
amp, str, tc, kn, nm,val	-	1	-	-	-	-	-
amp, str, tc, nitro, kn, nm, val	1	-	-	-	-	-	-
amp, str, tc, nitro, kn, nm, gent, val	-	1	-	-	-	-	-
TOTAL MULTIPLE RESISTANT	17	37	11	4	2	1	5

Numbers in brackets are number of strains tested.
Resistance to pen, nov and ery were omitted.

Evidence of R-plasmid mediated drug resistance, in the form of conjugal transfer frequencies of selected markers, is presented in Table 7. Potential donors were selected which were resistant to 2 or more antibiotics. Data are illustrated from selected experiments with *E. coli* Nal^r W3110 and various Nal^r *Klebsiella* as recipients.

TABLE 7. CONJUGAL TRANSFER FREQUENCIES OF DRUG RESISTANCE BY SELECTED *KLEBSIELLA* ISOLATES WITH *E. COLI* Nal^r W3110 OR OTHER Nal^r *KLEBSIELLA* AS RECIPIENTS

ISOLATE	ORIGIN	MARKERS DETECTED	MARKERS TRANSFERRED	FREQUENCY OF TRANSFER: TRANSCONJUGANTS/ RECIPIENT
		E. COLI W3110 AS RECIPIENT:		
C-2	Redwood	amp, val	amp	6.9×10^{-4}
			val	2.9×10^{-3}
			amp, val	4.2×10^{-3}
C-1	Redwood	amp, val	amp	8.5×10^{-4}
			val	8.1×10^{-4}
			amp, val	2.4×10^{-3}
BC 1738	Pulp mill	amp, nitro	-	-
E44.5-2	Redwood	amp, val	amp	1.2×10^{-6}
			val	6.0×10^{-6}
			amp, val	1.6×10^{-6}
V13	Redwood tank water	amp, str	str	6.1×10^{-4}
V5	Redwood tank water	amp, str	str	4.0×10^{-4}
V125	Potato	amp, nitro	nitro	1.6×10^{-6}
MH 10	Celery	amp, nitro	nitro	5.5×10^{-4}
SL 5	Human sputum	amp,str,tc nm,kn	str	2.0×10^{-2}
			amp	1.4×10^{-4}
			tc	2.5×10^{-2}
			kn	2.1×10^{-2}
			nm	2.1×10^{-2}

(continued)

TABLE 7. (continued)

ISOLATE	ORIGIN	MARKERS DETECTED	MARKERS TRANSFERRED	FREQUENCY OF TRANSFER: TRANSCONJUGANTS/ RECIPIENT
		E. COLI W3110 AS RECIPIENT:		
UT 465	Human blood	amp,str,fur tc	amp	5.1×10^{-5}
			str	2.6×10^{-7}
			fur	1.2×10^{-2}
			amp,nitro	2.3×10^{-4}
			str,nitro	2.5×10^{-2}
UG 14	Bovine Clinical	str, tc	str	1.0×10^{-2}
			tc	9.8×10^{-4}
			str, tc	4.3×10^{-6}
UG 28	"	str,tc,chl	str	2.9×10^{-5}
			chl	1.2×10^{-5}
			str,chl	1.2×10^{-5}
UG 6	unused bedding	str, tc	str	2.7×10^{-5}
			tc	5.8×10^{-4}
			str, tc	1.4×10^{-6}
UG 16	unused bedding	amp, str	-	-
			tc	4.2×10^{-7}
			str, tc	6.6×10^{-7}

KLEBSIELLA AS RECIPIENTS:

KLEBSIELLA RECIPIENT	ORIGIN	DONOR	ORIGIN	MARKER SELECTED	FREQUENCY OF TRANSFER: TRANSCONJUGANTS/ RECIPIENT
V-104A	Vegetable	UG 14	Bov. Clinical	str	7.6×10^{-4}
"	"	UG 28	Bov. Clinical	chl	5.9×10^{-4}
"	"	UG 34	Bov. Anal	val	4.6×10^{-4}
MH 15	Salad	UG 28	Bov. Clinical	chl	2.9×10^{-6}
"	"	UG 34	Bovine Anal	val	6.2×10^{-5}
"	"	UT 465	Human, blood	nitro	3.2×10^{-3}
"	"	SL 5	Human, sputum	kn	1.2×10^{-3}
				nm	1.0×10^{-3}

(continued)

TABLE 7. (continued)

KLEBSIELLA RECIPIENT	ORIGIN	DONOR	ORIGIN	MARKER SELECTED	FREQUENCY OF TRANSFER TRANSCONJUGANTS/ RECIPIENT
			KLEBSIELLA AS RECIPIENTS:		
PS 401LR	Bovine Mastitis	UG 28	Bov. Clinical	chl	1.1×10^{-2}
"	"	SL 5	Human, sputum	kn	3.9×10^{-5}
				nm	1.1×10^{-4}

Overall, the transfer of markers ranged from 10^{-2} to 10^{-8} transconjugants per recipient. In all, 31 potential Klebsiella donors were tested for transfer of antibiotic resistance and transconjugants were detected for 28 cultures.

Selected isolates that supported measurable transfer into E. coli were chosen as donors to various Klebsiella of environmental and medical origins. Depending upon the markers selected, Klebsiella isolates had various degrees of success as conjugal recipients. In the case of Str, Tc and the Kn-Km markers, transfer was a more frequent event when E. coli was the recipient. In the case of Val and Nitro, the frequencies were of the same magnitude, and Chl was usually mobilized more frequently into Klebsiella than into E. coli.

These data illustrate that R-plasmid bearing Klebsiella isolates from a variety of "natural" origins are able to mobilize drug resistance to either an E. coli or another Klebsiella recipient with frequencies similar to isolates of clinical origins. If these Klebsiella were to become involved in colonizing compromised hosts, such cultures would have an obvious selective advantage accompanying antibiotic therapy.

COMPARATIVE PATHOGENICITY OF THE KLEBSIELLA

The major point of these studies was to compare and contrast in a controlled system the virulence properties themselves among a set of Klebsiella isolates derived from various habitats and differing in known phenotypic properties. In all cases of mouse death following injection, the inoculated Klebsiella was recovered from several organs and was also present in the blood stream. Death was therefore confirmed to be due to the inoculated organism and was the result of the relative invasiveness of the test culture and the ability of the culture to overcome the normal host defenses.

Mean LD_{50} values for 97 Klebsiella and for 12 E. coli isolates are illustrated in Table 8. The mean LD_{50} values for Klebsiella of all origins

TABLE 8. LD_{50} OF *KLEBSIELLA* AND *E. COLI* ISOLATES DETERMINED BY INTRAPERITONEAL INJECTION IN MICE

CULTURE	SOURCE	NUMBER OF ISOLATES	MEAN LD_{50}[a] (cells per ml)	RANGE (cells per ml)
Klebsiella spp.	Mastitis	19	1.5×10^4	3.5×10^1 to 4.9×10^5
	Clinical	18	4.6×10^4	5.3×10^3 to 6.0×10^5
	Environmental	60	4.2×10^4	1.0×10^3 to 7.9×10^5
E. coli	Clinical	6	2.8×10^4	4.5×10^3 to 2.9×10^5
	Finished Drinking Water	6	2.5×10^5	6.2×10^4 to 2.2×10^6

[a]Geometric mean based on natural log values.

ranged from 1.5×10^4 to 4.6×10^4 cells. Statistical tests indicate no significant difference ($\alpha = .05$) in the mean values for Klebsiella derived from mastitis, clinical, or environmental origins. The mean LD_{50} for E. coli of human clinical origin is not significantly different from mean values for Klebsiella from any source. On the other hand, E. coli from finished drinking water had a significantly higher mean LD_{50} than both the clinical E. coli and all Klebsiella. In a study also conducted in this laboratory, the LD_{50} values for other gram-negative bacteria (Enterobacter, Erwinia, Salmonella, Aeromonas, Pseudomonas and Spirillum) were 100- to 1,000-fold higher, indicating a significantly lower virulence for mice than Klebsiella (32).

Although the Klebsiella LD_{50} values have a 2-log range for clinical and environmental isolates and a 4-log range for mastitis cultures, the central tendency for all groups is within the same log 4.0 to 4.9 range (Figure 4).

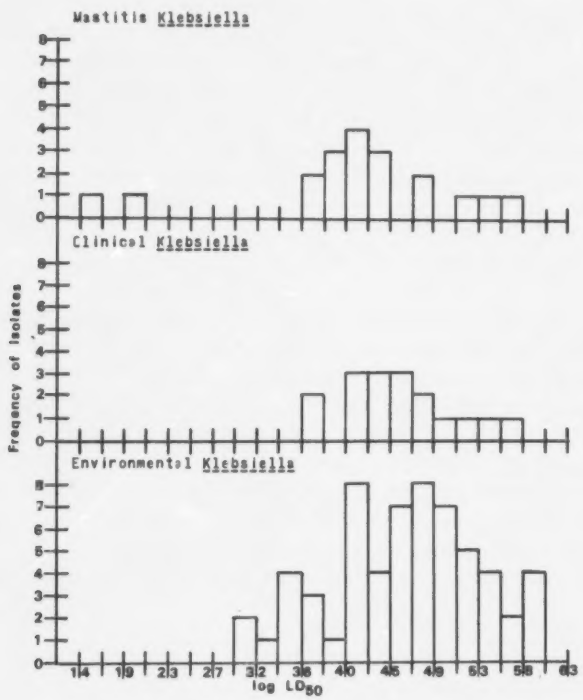


Figure 4. LD_{50} frequency distribution in mice for Klebsiella isolated from various environments.

The mean LD_{50} values for various groups of the Klebsiella isolates (based on FC response and indole production, K. oxytoca) are presented in Table 9. Although the calculated mean LD_{50} values range from 1.1×10^4 to 6.0×10^4 cells and sample size varied from 3 to 23 cultures, statistical tests again indicate no significant difference in the mean LD_{50} values for any of the various groups.

The environmentally derived Klebsiella were sub-divided by habitat of origin to possibly ascertain whether any differences in mean LD_{50} values could be detected (Table 10). Two interesting correlations were revealed. First, there is no significant difference between the mean LD_{50} values of Klebsiella isolated from finished drinking water, from bovine associated environments, and from bovine-areas compared to those Klebsiella of clinical origins. Second, the finished drinking water-derived Klebsiella have a significantly different mean LD_{50} (more virulent) than the drinking water derived E. coli but are indistinguishable in virulence from the clinical E. coli. Klebsiella and E. coli isolated from finished drinking water were generally obtained from the same water samples (24).

The overall conclusion of this comparative survey illustrates that a Klebsiella from any source is equally pathogenic in the animal test system. Unlike the Klebsiella isolates, there is a difference in virulence between E. coli from different sources. This indicates that E. coli, which can also be an opportunistic human pathogen (33), may have diminished virulence upon entry into the environment. A similar observation has also been made for Salmonella (32). Klebsiella entering the environment may have no such decrease in virulence as a recent st3dy with three Klebsiella of pathogenic origin (31) showed no change in LD_{50} values after 100 generations of growth in sterile pulp mill effluent.

The sanitary significance of FC-negative Klebsiella may be enhanced due to the finding that no difference in mouse pathogenicity was observed between the FC-positive and FC-negative isolates, regardless of habitat of origin. It must also be remembered that the indole positive (K. oxytoca) isolates of Klebsiella are also found as human and animal pathogens (27,30) and are present in the feces of warm-blooded animals (30,35). In addition, the importance of environmentally-derived Klebsiella is based on their opportunistic pathogenicity and is now underscored by the fact that drinking water-derived FC positive and FC negative Klebsiella have statistically lower LD_{50} values than drinking water-derived FC positive E. coli.

KLEBSIELLA PERSISTANCE IN MOUSE INTESTINAL TRACT FOLLOWING INGESTION OF CONTAMINATED DRINKING WATER

Klebsiella within the intestinal tract can be a direct cause of nosocomially acquired infection (36,37) and intestinal persistance or colonization results from ingestion of contaminated foods and liquids (33,36). The previous section on Klebsiella LD_{50} levels illustrated that, given the chance to enter the intraperitoneal cavity, Klebsiella from any environment or of

TABLE 9. LD_{50} of *K. PNEUMONIAE* AND *K. OXYTOCA* DETERMINED BY INTRAPERITONEAL INJECTION IN MICE

CULTURE	SOURCE	NUMBER OF ISOLATES	MEAN LD_{50}[a] (cells per ml)	RANGE (cells per ml)
FC[b] Positive *K. pneumoniae*	Mastitis	11	1.6×10^4	3.5×10^1 to 4.9×10^5
	Clinical	11	3.9×10^4	5.8×10^3 to 6.0×10^5
	Environmental	14	5.5×10^4	1.0×10^3 to 7.9×10^5
FC Negative *K. pneumoniae*	Mastitis	3	1.5×10^4	6.5×10^3 to 2.5×10^4
	Clinical	3	6.0×10^4	1.6×10^4 to 3.3×10^5
	Environmental	23	4.5×10^4	1.3×10^3 to 7.5×10^5
K. oxytoca[c]	Mastitis	5	1.1×10^4	4.6×10^3 to 2.4×10^4
	Clinical	4	5.8×10^4	5.3×10^3 to 5.1×10^5
	Environmental	23	3.7×10^4	3.4×10^3 to 6.3×10^5

[a]Geometric mean based on natural log values.

[b]Fecal Coliform.

[c]*K. oxytoca* are, by definition, FC negative (21,22).

TABLE 10. LD_{50} OF ENVIRONMENTAL KLEBSIELLA ISOLATES DETERMINED BY INTRAPERITONEAL INJECTION IN MICE

SOURCE	NUMBER OF ISOLATES	MEAN LD_{50}[a] (cells per ml)	RANGE (cells per ml)
Finished Drinking water (Redwood reservoirs)	28	5.2×10^4	3.8×10^3 to 6.9×10^5
Vegetables[b]	8	1.4×10^5	4.1×10^3 to 7.9×10^5
Redwood sawdust and chips	6	9.1×10^4	6.0×10^4 to 4.2×10^5
Bovine-associated[c]	9	1.5×10^4	1.0×10^3 to 1.3×10^5
Bovine-area[d]	9	1.3×10^4	1.6×10^3 to 1.3×10^5

[a]Geometric mean based on natural log values.

[b]Isolates from potatoes (5), carrots (2), and Swiss chard (1).

[c]Isolates from bovine mouth (5), anal area (1), and udder (3).

[d]Isolates from unused bedding (2), used bedding (2), and drinking water (5) in bovine stall areas.

any biotype has no significant difference in levels of virulence for mice. The purpose of these studies was to determine what levels of ingested Klebsiella are necessary to persist in the mouse intestinal tract and how such orally administered levels compare with known concentrations of Klebsiella in drinking water and other environments. In this manner it becomes possible to compare the ability of known levels of pathogenic isolates to survive/persist in the intestines compared to environmentally-derived isolates whose prior ecological history is unknown. Fig. 5 summarizes the percentage of mice excreting Klebsiella after oral ingestion while Fig. 6 illustrates the average density of Klebsiella in fecal pellets.

Percent animal carriers reach a constant amd maximum value by day 2 which persists until the contaminated water is replaced with municipal water on day 6. The maximum percent animal carriers varied with the Klebsiella densities per ml of water (10^3, 10^1, and 10^{-1}) and resulted in percent animal carriers of 80%, 63%, and 8%, respectively. It should be noted that mice provided 10^{-1} Klebsiella/ml (10/100 ml) were only ingesting about one viable Klebsiella per 24 hr taking into consideration some Klebsiella regrowth in the water. Nevertheless it was still possible to detect Klebsiella in some two of 48 control mice and nine of 48 mice administered antibiotics.

With every dose level studied, there was a pronounced increase in percent animal carriers and fecal cell densities accompanying antibiotic

treatment. When the drinking water contained Nal (40 ug/ml) and Str (500 ug/ml) the respective animal carrier rates increased to 95%, 78%, and 19% for the three-dose levels (Fig. 5).

Fecal densities for Klebsiella provided at 10^1 and 10^3/ml of drinking water are presented in Fig. 6. With no antibiotic stress, Klebsiella are lost from the intestine within 4 days after removal of contaminated drinking water containing 10^1 cells/ml. On the other hand Klebsiella persist at an average density of 300/gm feces 8 days after removal from contaminated drinking water when antibiotics are continuously supplied along with a single CP injection (given on day 3). In similar experiments, fecal densities are correspondingly greater at the 10^3 Klebsiella/ml dose level.

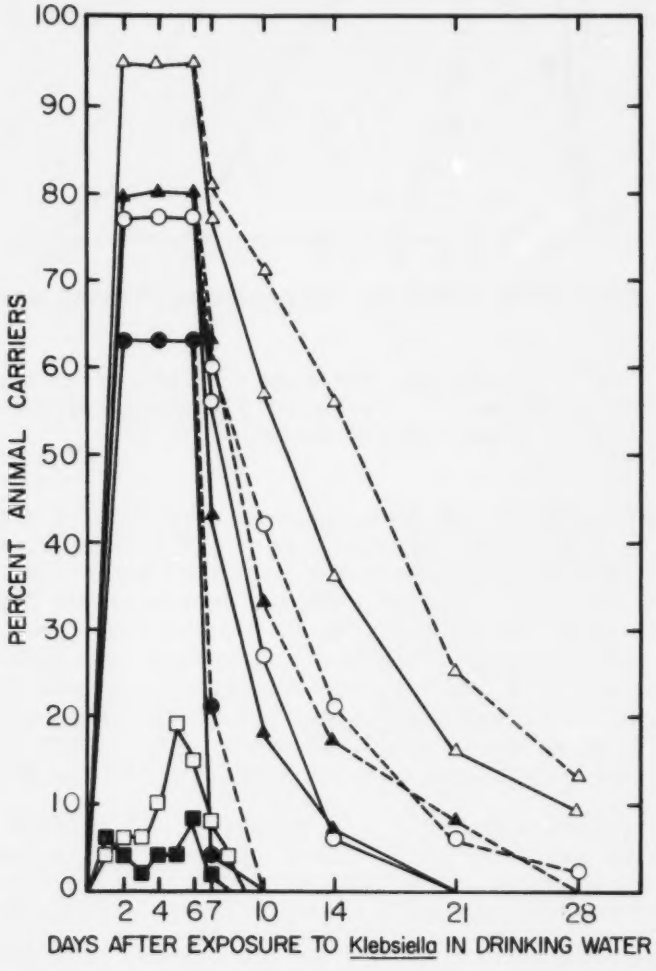


Figure 5. Percentage of mice excreting Klebsiella after ingestion of contaminated drinking water. Symbols represent levels of Klebsiella/ml drinking water: Δ, Δ, 10^3; 0, 0, 10^1; , , 10^{-1}. Open symbols with antibiotics, closed, without antibiotics in drinking water; dashed lines CP injected day 3, solid lines, no CP. Klebsiella present in drinking water through day 6.

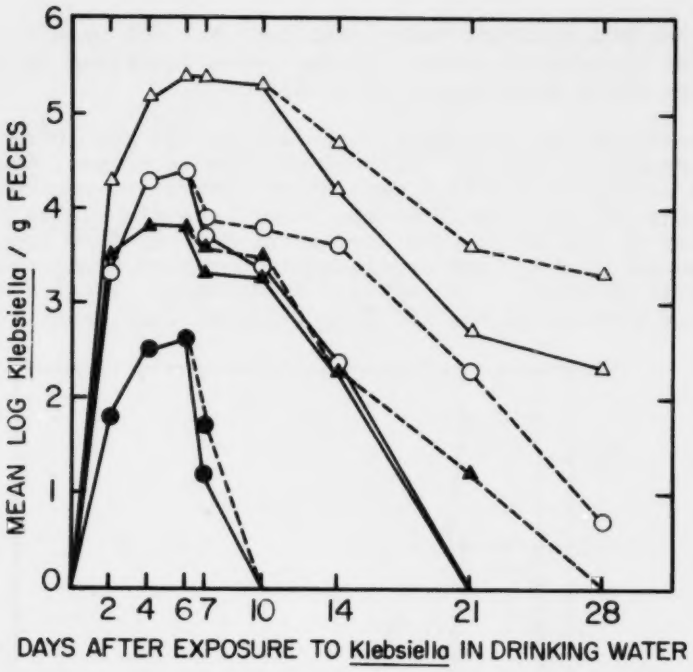


Figure 6. Mean log of Klebsiella per gram of mouse feces after ingestion of contaminated drinking water. Levels of Klebsiella/ml drinking water: Δ, Δ, 10^3; 0, 0, 10^1. Other symbols as in Figure 5.

Klebsiella recovered from feces of even control mice (no stress) were in excess of the orally ingested number. For example, based on actual viable counts in the drinking water and volume of water consumed, mice provided the 10^1 cells/ml actually ingested approximately 320 total cells over the first 4 day period. On day 4, the level recovered equalled 320 cells, or every Klebsiella ingested was recovered. After an additional 48 hr some 240 cells were ingested and 400 Klebsiella were recovered in one sample. With oral antibiotics and ingestion of the same number of Klebsiella, 20,000 Klebsiella were recovered per gm feces on both days and 4 and 6 demonstrating considerable multiplication of Klebsielal in the intestinal tract.

A distinction among Klebsiella in terms of both percent animal carriers (Fig. 7) and fecal densities (Fig. 8) was observed when cultures were divided by fecal coliform response and K. oxytoca species. Although each of the 3 groups contained only 2 Klebsiella isolates (one clinical, one environmental) the data was clear in that fecal coliform positive isolates colonized the greatest percent of animals and achieved the highest cell densities/gm feces. Fecal coliform negative Klebsiella performed the poorest in both respects while the indole producing K. oxytoca were intermediate.

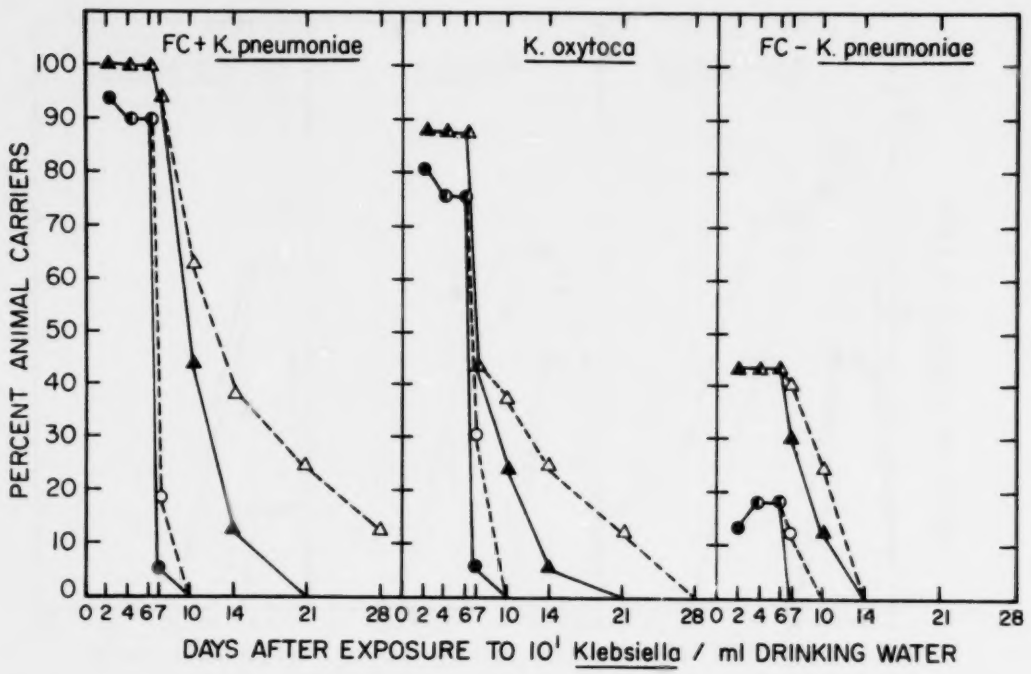


Figure 7. Percentage of mice excreting various species/biotypes of Klebsiella after ingestion of contaminated drinking water containing 10^1 cells/ml of FC+ K. pneumoniae, K. oxytoca, or FC- K. pneumoniae. Δ, Δ, with antibiotics, 0, 0, without antibiotics in drinking water. Open symbols, CP injected on day 3, closed symbols, no CP. Klebsiella present in drinking water through day 6.

The results (Figs. 5 and 6) illustrate that ingestion of 10^{-1} or greater Klebsiella/ml over a short period can result in temporary intestinal carriage at levels up to 100-times the ingested dose. The results of these studies using mice probably should not be extrapolated to the human population although some experimental data from humans is relevant. In one experiment it was found that approximately 10^5 Klebsiella ingested by humans would result in a temporary fecal persistance for 3-4 days at levels of 10^3/gm. These observations are compatable with the results of other studies in this laboratory using a single oral administration of Klebsiella to mice.

Fecal coliform response and species, not habitat of origin, had a significant effect on the ability of a Klebsiella to persist in the mouse intestinal tract (Figs. 7 and 8). The fecal coliform positive Klebsiella were more effective than K. oxytoca and the latter were more effective than

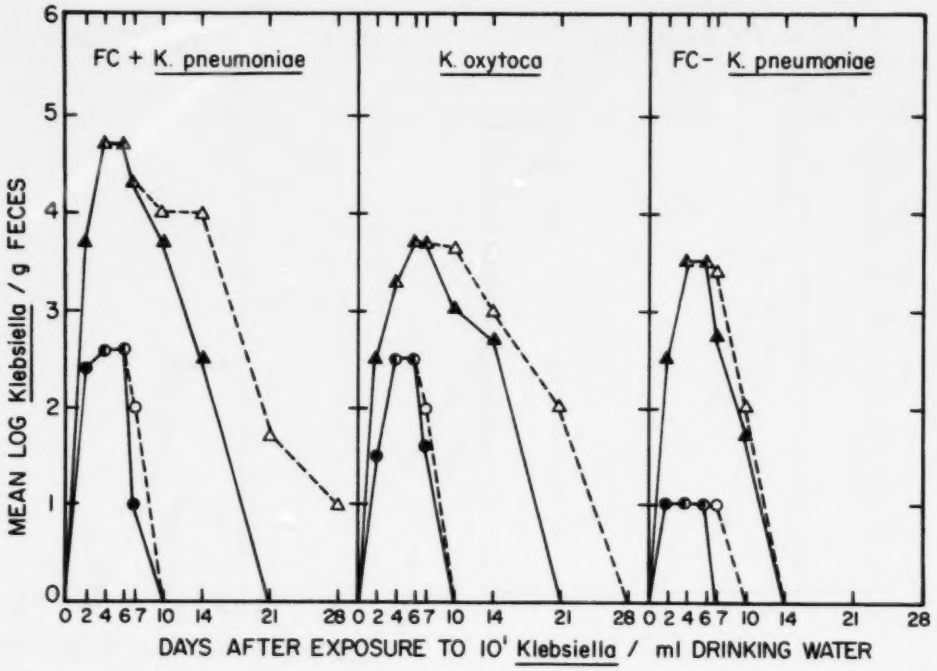


Figure 8. Mean log of Klebsiella species/biotype per gram mouse feces after ingestion of contaminated drinking water. Symbols as in Figure 7.

fecal coliform negative cultures. While oral antibiotics significantly increased the persistance levels of fecal coliform negative cultures, levels attained were still below those for the other two groups. These results have important implications concerning the ecological distributions of the various types of Klebsiella. Greater than 85% of the known pathogenic Klebsiella tested for fecal coliform response have been positive in this test. K. oxytoca accounted for some 14% of the pathogenic cultures and the fecal coliform negative specimens for about 1%. Isolates of Klebsiella from human and animal feces are also in roughly the same proportions (7,30,38). Among the environmentally-derived Klebsiella isolates, the reverse order has been found with the fecal coliform negative Klebsiella predominating.

Fecal coliform positive Klebsiella from clinical origins will regrow in botanical environments, including drinking water emanating from redwood reservoirs, pulp mill effluents, surfaces of fresh vegetables, and in aqueous extracts of sawdust (31,39). Since fecal coliform positive Klebsiella represent the majority of Klebsiella responsible for infections and they are also the type most likely to persist in the intestinal tract, their isolation should be regarded as the most potentially significant health hazard. The

indole positive, fecal coliform negative K. oxytoca should also be similarly viewed. They are the primary agents of infections in animals and humans and are also carried in feces. The significance of these Klebsiella might be overlooked in various environmental samples since they would appear as total and not fecal coliforms.

THE 3,785 LITER (1,000 GAL) EXPERIMENTAL RESERVOIR

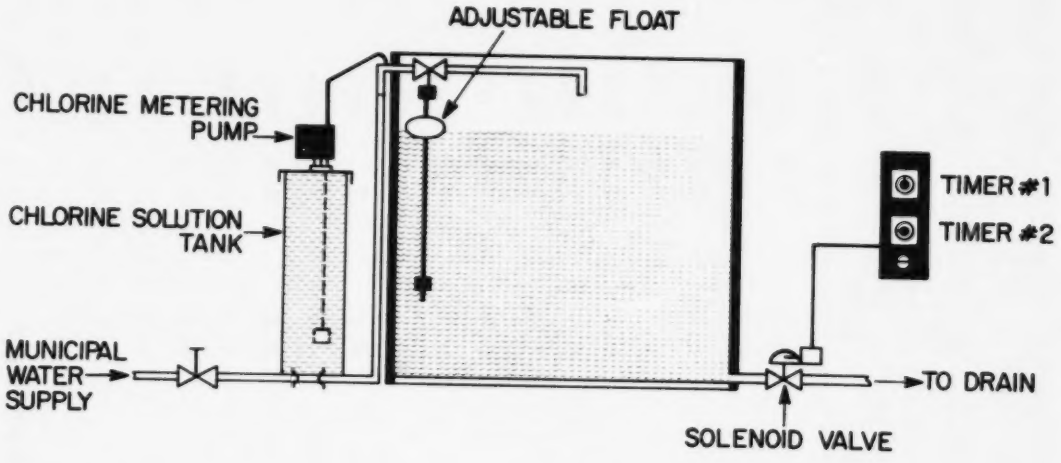


Figure 9. Diagram of experimental 3,785 liter (1,000 gal) reservoir used to test design modifications in redwood water storage reservoirs.

Based on findings drawn from the field surveys in Oregon, design modifications were made on a 3,785 liter experimental reservoir which was constructed for research purposes (Fig. 9). An automated chlorine metering system was employed, and an inlet water pipe separate from the outlet was installed at the top of the tank. The experimental rationale was based on an attempt to maintain an available chlorine residual and adequate water mixing to maintain coliform-free water at various average retention times. Regular monitoring (usually daily) of physical parameters of the water was performed. Samples of water were frequently examined for bacteriological quality, and materials from the interior surfaces of staves was scraped at intervals and examined for coliforms and other bacteria. Table 11 summarizes studies over a 20 mo. period on the experimental reservoir.

TABLE 11. PHYSICAL AND MICROBIOLOGICAL PARAMETERS IN THE 3,785 LITER EXPERIMENTAL REDWOOD TANK

Month of operation	Retention time (days)	Number of days sampled	Free Chlorine at inlet (ppm)	Free Chlorine at outlet (ppm)	pH	OD_{600}	Average total count/ml	Stave scraping
1	2[a]	7	1.7	0.3	7.0	.009		
1	4[a]	9	4.9	0.1	7.1	.018	225	NCD[b]
1	8[a]	6	11.5	0.2	7.4	.020	1040	
2	8[a]	13	23.3	0.5	7.0	.009	123	
2	12[a]	11	18.2	0.1	7.0	.012	15	NCD
3	12[a]	6	30.0	0.1	7.0	.008		
3	1	20	8.6	3.7	7.1	.001	3	
4	1	26	2.1	0.9	7.1	.001	>1000	NCD
5	1	5	1.9	0.2	7.0	.001	>1000	
5	2	4	3.0	0.4	7.1	.003	>2000	
5	4	21	3.9	0.7	7.1	.001	>2000	NCD
6	4	14	5.1	0.4	7.0	.001	10	
7	8	19	8.2	0.3	6.8	.001	95	
8	8	28	8.6	0.5	7.0	.000	<10	NCD
9	8	24	5.8	0.2	7.0	.001	25	
10	8	27	5.8	0.5	7.1	.000	<10	NCD
11	8	28	4.2	0.6	7.3	.000	<10	

(continued)

TABLE 11. (continued)

Month of operation	Retention time (days)	Number of days sampled	Free Chlorine at inlet (ppm)	Free Chlorine at outlet (ppm)	pH	OD_{600}	Average total count/ml	Stave scraping
12	8	19	3.3	0.4	7.2	.000	40	NCD
12	12	7	3.9	0.3	7.2	.000		
13	12	28	3.0	0.4	7.2	.000	20	
14	12	23	2.7	0.5	7.3	.000	<10	*Klebsiella pneumoniae* FC[c]-
15	12	23	1.5	0.4	7.2	.000	21	NCD
16	12	19	0.5	0.1	7.1	.000	2060	
17	12	7	0.5	0.2	7.2	.000	1090	
18	12	7	0.5	<0.1	7.2	.000	860	
19	12	6	0.5	<0.1	7.0	.000	10	
20	12	3	0.5	<0.1	7.0	.000	48	NCD

[a]Tank operating at 20 percent capacity.

[b]NCD - no coliforms detected.

[c]FC-; fecal coliform negative.

Total bacterial counts in the outlet water ranged from 2,000/ml to less than 10 per ml during the study period. The counts were generally in the 10-50/ml range when an adequate free chloring residual of 0.2-0.4 ppm was maintained. Chlorine additions from the metering pump were terminated in the 16th month and municipal water with 0.3-0.5 ppm became the only source of chlorine. This resulted in a lowering in the chlorine residual at the outlet and a temporary increase in total counts to 2,000/ml, which decreased to less than 50/ml by the 19th mo. of operation. Non-coliform isolates were identified and found to consist of: Pseudomonas spp., P. fluorescens, Moraxella, Xanthomonas, Bacillus, Micrococcus, and one Staphylococcus epidermidis.

Coliforms were detected in only one of the eight experiments in which the interior surfaces of the staves were scraped and examined. Only five of the eighteen staves sampled yielded coliforms which were identified as fecal coliform negative K. pneumoniae. Subsequent stave scrapings failed to detect any coliforms. At no point were coliforms detected in water emanating from the tank, even when volumes of 1,000 ml were filtered and assayed by the MF technique.

Two factors were confirmed to be important in maintaining a free chlorine residual in the water of the redwood reservoir. As the tank aged, the chlorine demand decreased (Table 11). Thus, from the 7th to the 15th month (8 to 12 day retention), the amount of chlorine needed to maintain a free residual of 0.4 ppm decreased from 8.2 ppm to 1.5 ppm. Retention time also has an important influence on chlorine residuals. In the 4th month of operation (1 day retention), 2.1 ppm at the inlet maintained a residual of 0.9 ppm, but 5.1 ppm was needed on a 4 day retention to maintain a lower residual of 0.4 ppm. Also, on an 8 day retention, 8.2 ppm in the inlet water was required to maintain 0.3 ppm in the outlet water.

The presence of colored material (tannins) in the reservoir water (Optical density measurements, Table 11) also indicates the presence of a variety of organic matter. To test whether this material contains growth promoting substances for coliforms, a strain of K. pneumoniae was inoculated into dechlorinated filter sterilized samples of tank water. Of 15 water samples tested, five supported growth of the Klebsiella to levels of 10^5 to 10^7/ml (Table 12). Since all glassware was acid washed, the only source of nutrients was from the redwood used to construct the tank. No correlation was found between those samples that supported growth and those that did not.

These studies illustrate that, with routine adjustments of design, redwood water reservoirs can be operated without coliform contamination. However, there are certain factors that must be taken into consideration, which, if not adhered to, can result in high levels of coliform contamination as recorded in the field surveys. These factors include maintaining an adequate chlorine residual in the reservoir and throughout the distribution system, maintaining short retention times of water in the reservoir, and a design which promotes adequate water circulation within the reservoir. Even though the experimental 3,785 liter reservoir was constructed and maintained under the best known conditions, K. pneumoniae was found on one occasion to be colonizing the staves. This observation, coupled with their

TABLE 12. VIABLE COUNTS AT 35C OF KELBSIELLA INCUBATED IN REDWOOD TANK WATER

Date sample collected	0 hr	48 hr	96 hr
2/26/75	9.0×10^1	1×10^1	1×10^1
4/5/75	7.0×10^1	1×10^1	1×10^1
10/24/75	4.5×10^1	3.0×10^2	3.0×10^2
11/13/75	2.3×10^0	1×10^1	1×10^1
12/18/75	6.0×10^1	1×10^1	1×10^1
2/7/76	5.5×10^1	1×10^1	1×10^1
6/30/76	2.3×10^0	3.7×10^5	1.6×10^6
7/29/76	2.3×10^0	1.5×10^6	1.5×10^6
9/15/76	7.0×10^1	1×10^1	1×10^1
10/10/76	8.0×10^1	1.3×10^7	9.4×10^6
11/19/76	4.0×10^1	5×10^0	1×10^1
1/11/77	2.3×10^0	2.5×10^6	1.8×10^6
2/10/77	8.0×10^1	4.2×10^6	5.0×10^6
3/10/77	9.5×10^1	1×10^1	1×10^1
5/18/77	6.0×10^1	1×10^1	1×10^1
municipal water	7.5×10^1	1×10^1	1×10^1
double distilled water	9.5×10^1	1×10^1	1×10^1

demonstrated ability to regrow in the tank water, clearly enhances the necessity to maintain a free chlorine residual throughout the system at all times.

The amount of chlorine required to maintain a free residual in the outlet water actually depends upon a combination of 3 interrelated phenomena. The combination of a new reservoir and lengthy retention time (7 days or more) and poor water circulation each individually aggravate the chlorine demand. In order to maintain a free residual at the outlet with adequate water mixing within the experimental reservoir. it required approximately 1 ppm free chlorine in the inlet water for each day of water retention during the interval of 3-10 months. The introduction of water with an initial chlorine residual of 0.3 ppm is therefore clearly inadequate.

During the first 3 months of operation it was necessary to use 15-20 ppm and more chlorine to maintain a residual on an 8 to 12-day retention period. Therefore, if possible, it would be highly beneficial to install and bring on line a new reservoir several months prior to the actual serving of finished drinking water to the consumer. Prior to the preservice period, it is recommended that the manufacturer's guide for a 24 h soda ash soak to leach tannins and other water soluble material be extended to 7 days. Optical density readings taken during the second treatment of 7 days with the experimental reservoir showed that considerable additional material was leached from the wood. After this treatment, however, the odor and color of the water became quite acceptable and optical density reading demonstrated no significant difference compared with finished municipal water.

During the preservice period after the soda ash treatment, the reservoir should not contain unchlorinated, stagnant water, but rather, should operate in some intermittant fashion to drain out nutrients which continue to become leached from the wood. Also a chlorine residual should be maintained to prevent slime accumulation on the stave surfaces. If slime does accumulate, it should be scrubbed from the wooden surfaces so that it cannot slough off and enter the distribution system at later times.

Finally, all redwood reservoirs should utilize a plumbing arrangement at the top of the tank for inlet water that sprays the water evenly to all sides. The outlet fixture should be located at or near the tank bottom. This promotes mixing and the spraying of water onto the upper staves, promotes aging of wood and leaches out nutrients from areas that might not become immersed below the water line. By adjusting the level of floats, the volume of water in the reservoir can be matched with the daily anticipated usage of water such that retention times can be maintained to a minimum.

In summary, redwood tanks can be a satisfactory means to store water for drinking purposes, providing a few routine guidelines are followed and a monitoring schedule of chlorine residuals and bacteriological sampling is maintained. This is especially important for new redwood tanks, where the likelihood of coliform contamination is most severe. When properly managed, however, redwood water systems can be expected to provide potable drinking water throughout the life of the tank.

ASSOCIATION OF *KLEBISELLA* WITH REDWOOD USED TO CONSTRUCT RESERVOIRS

In the previous section, evidence was presented to document the presence of *Klebsiella* and *Enterobacter* emanating from wooden reservoirs containing finished drinking water. In this section, evidence will be presented to demonstrate that the origin of these coliforms can be from the wood used to construct reservoirs. Evidence has been obtained in field surveys which demonstrates that *E. coli* is also occasionally present in the finished drinking water but its origin should be considered to be due to more recent fecal contamination of the water distribution system and not from the redwood *per se*.

Table 13 illustrates the results of 3 experiments which examines the bacterial content of liquid aseptically extracted from redwood heartwood (HW) and sapwood (SW). The results indicate that not only are high levels of standard plate count bacteria present, but high levels of total coliforms as well. Levels of total coliforms ranged from 3.2×10^2 to 1.4×10^6/ml of liquid extracted. High total coliform counts also correspond with high levels of total standard plate counts.

All coliforms identified were of the Klebsiella and Enterobacter genera. E. agglomerans was the only coliform identified from the redwood SW tested in Experiment 1. Samples in Experiment 2 were incubated at higher temperatures to reduce the numbers of Enterobacter and enhance the isolation of Klebsiella. Klebsiella species were in fact recovered from all samples having detectable total coliforms. In Experiment 3 Klebsiella were also isolated from redwood samples although incubation was at 35°C. No fecal coliforms were detected from any wood sample by plating onto mFC agar.

Liquid expressed from other types of wood was also examined for bacterial content (Table 14). With the exception of cedar, all woods tested were SW and had levels of standard plate counts comparable to those found from redwood. All wood except cedar also had recoverable total coliforms but counts were 10- to 100-times less than in the redwood extracts. Coliforms identified were essentially the same as those from redwood, with the additional isolation of Citrobacter freundii.

Contact impressions of freshly exposed wood surfaces (Table 15) gave results parallel to the total coliform counts in liquid expressed from the same wood samples (Table 13). All wood types from which total coliforms were obtained via pressing also had total coliforms present on the inner surfaces.

Table 16 presents the results of contact impressions made from successive serial cuts through two redwood SW samples having confluent coliform growth (Table 15). The results generally demonstrate that coliforms are present throughout the entire depths of the block.

Scanning electron micrographs (SEM) of wide ring count SW sections (Figs. 10 to 12) taken at increasing magnifications clearly demonstrate the presence of several types of bacteria within the wood structure. Further evidence that the bacteria detected in these studies are actually within the wood itself is the fact that no bacteria were detected on the surface sterilized saw blade, table top, or other equipment after surface sterilization.

The three types of coliforms identified in these studies from all types of wood, Klebsiella, Enterobacter, and C. freundii, have few biochemical test differences from previously reported studies (2,4). The only test reaction with possible significance was the increase in numbers of Enterobacter spp. able to ferment inositol. Forty-eight percent of E. agglomerans and 33% of E. cloacae fermented inositol, as compared to reference values of 18% and 22% (2), respectively, for the two species. All of the Klebsiella isolates fermented inositol.

TABLE 13. BACTERIAL DENSITIES IN LIQUID EXPRESSED FROM REDWOOD AND COLIFORMS IDENTIFIED

Type of wood	No. of samples	Bacterial Counts per ml		Coliforms Identified
		Total Bacteria[a]	Total Coliform[b]	
Experiment 1. Cutwood samples 1-2 months old				
Sinker HW	2	3.3×10^5	NG	
Heavy Stain HW	1	1.3×10^3	NT	
Young Growth HW	2	NG	NG	
Wide Ring Count SW	2	4.5×10^6	TNTC	Enterobacter agglmerans
Heavy SW	2	1.7×10^6	2.0×10^3	E. agglomerans
Experiment 2. Freshly cut wood from saw mill				
Trim green HW	1	3.5×10^4	NG	
HW	2	1.7×10^4	NG	
HW and SW	1	5.0×10^5	4.0×10^3	Klebsiella pneumoniae
HW and SW chips	2	1.3×10^5	2.0×10^3	K. pneumoniae
HW and SW	1	1.0×10^5	5.0×10^2	K. pneumoniae, K. oxytoca
HW and SW	1	1.30×10^5	3.0×10^4	K. pneumoniae, E. cloacae, E. agglomerans
SW	1	NG	NG	
Experiment 3. Freshly cut wood samples from a log pile.				
Bottom log, HW	3	5.0×10^2	NG	
Bottom log, SW	3	1.4×10^7	1.4×10^6	E. agglomerans

(continued)

TABLE 13. (continued)

Type of wood	No. of samples	Bacterial Counts per ml		Coliforms Identified
		Total Bacteria[a]	Total Coliform[b]	
3rd cut, HW	3	NG	NG	
3rd cut, SW	3	1.3×10^6	4.5×10^4	K. pneumoniae, E. agglomerans, E. sp.
Top log, HW	3	1.0×10^2	NG	E. sp.
Top log, SW	3	5.7×10^3	3.2×10^2	K. pneumoniae, E. agglomerans

HW-Heartwood; SW-Sapwood; NG-No growth; NT-Not tested; TNTC-Too numerous to count.

[a]Average results after 48 hr of incubation on nutrient agar at 30°C, except for experiment 2 which was incubated at 37°C.

[b]Average results after 24 hr of incubation on m-Endo agar LES at 35°C, except for experiment 2, which was incubated at 37°C.

TABLE 14. BACTERIAL DENSITIES IN LIQUID EXPRESSED FROM NON-REDWOOD AND COLIFORMS IDENTIFIED

Type of wood	No. of samples	Bacterial Counts per ml			Coliforms Identified
		Total Bacteria[a]	Total Coliform[b] MPN[c]	m-Endo[d]	
Cedar	2	$<1.0 \times 10^1$	<2.0	0	Enterobacter agglomerans
Cottonwood SW	2	3.8×10^5	6.5×10^1	0	E. agglomerans, Citrobacter freundii
Douglas fir, freshly cut SW	3	4.5×10^2	0.9	0	E. agglomerans
Douglas fir, racked SW	2	7.0×10^5	1.3	0	Klebsiella pneumoniae, E. agglomerans
Hemlock SW	2	2.0×10^6	2.0	5	K. pneumoniae, E. agglomerans C. freundii[e]

SW-Sapwood; MPN-Most probable number.

[a]Results after 48 hr incubation at 30°C on nutrient agar.

[b]No fecal coliform were detected.

[c]Average of confirmed counts after 24 hr incubation at 35°C on EMB agar.

[d]Average results using m-Endo agar LES after 24 hr incubation at 35°C.

[e]Isolated as a green-sheened colony on m-Endo agar LES after 48 hr incubation.

TABLE 15. COLIFORMS RECOVERED FROM CONTACT IMPRESSIONS OF FRESHLY EXPOSED WOOD SURFACES

Type of Wood	No. of Samples	No. of Coliforms[a] 24 hr	48 hr	Coliforms Identified
Redwood, HSW	2	TNTC	TNTC	Enterobacter agglomerans
Redwood, WSW	2	TNTC	TNTC	E. agglomerans
Cedar	2	NG	NG	
Cottonwood, SW	2	NG	9	Citrobacter freundii
Douglas fir, freshly cut SW	6	E. agglomerans		
Douglas fir, racked SW	4	NG	10	E. agglomerans, C. freundii
Hemlock, SW	2	NG	1[b]	C. freundii

HSW-Heavy sapwood; WSW-Wide range count sapwood; SW-Sapwood; TNTC-Too numerous to count; NG-No growth.

[a]Reported as average number of green-sheened colonies appearing on m-Endo agar LES at 35°C.

[b]Number of colonies on only impression plate showing growth.

TABLE 16. COLIFORMS RECOVERED FROM CONTACT IMPRESSIONS OF SERIAL CUTS THROUGH REDWOOD[a]

Type of Redwood	No. of Samples	Surface Examined	No. of Coliforms[b] 24 hr	48 hr
Heavy Sapwood	2	upper surface	TNTC	TNTC
		2[c]	12	150
		3	10	150
		4	NG	100
		center	NG	100
		6	NG	100
		7	15	75
		8	30	75
		lower surface	TNTC	TNTC
Wide Ring Count Sapwood	2	upper surface	TNTC	TNTC
		2	TNTC	TNTC
		center	TNTC	TNTC
		4	TNTC	TNTC
		5	TNTC	TNTC
		lower surface	TNTC	TNTC

TNTC-Too numerous to count; NG-No growth.

[a]Block roughly 8 in.^{3} were cut in 1/4 in. slices.

[b]Reported as average number of green-sheened colonies appearing on m-Endo agar LES at 35°C.

[c]Number refers to position of cut section from upper surface.

In all cases, E. agglomerans was the only coliform identified and was present in all of the wood sections.

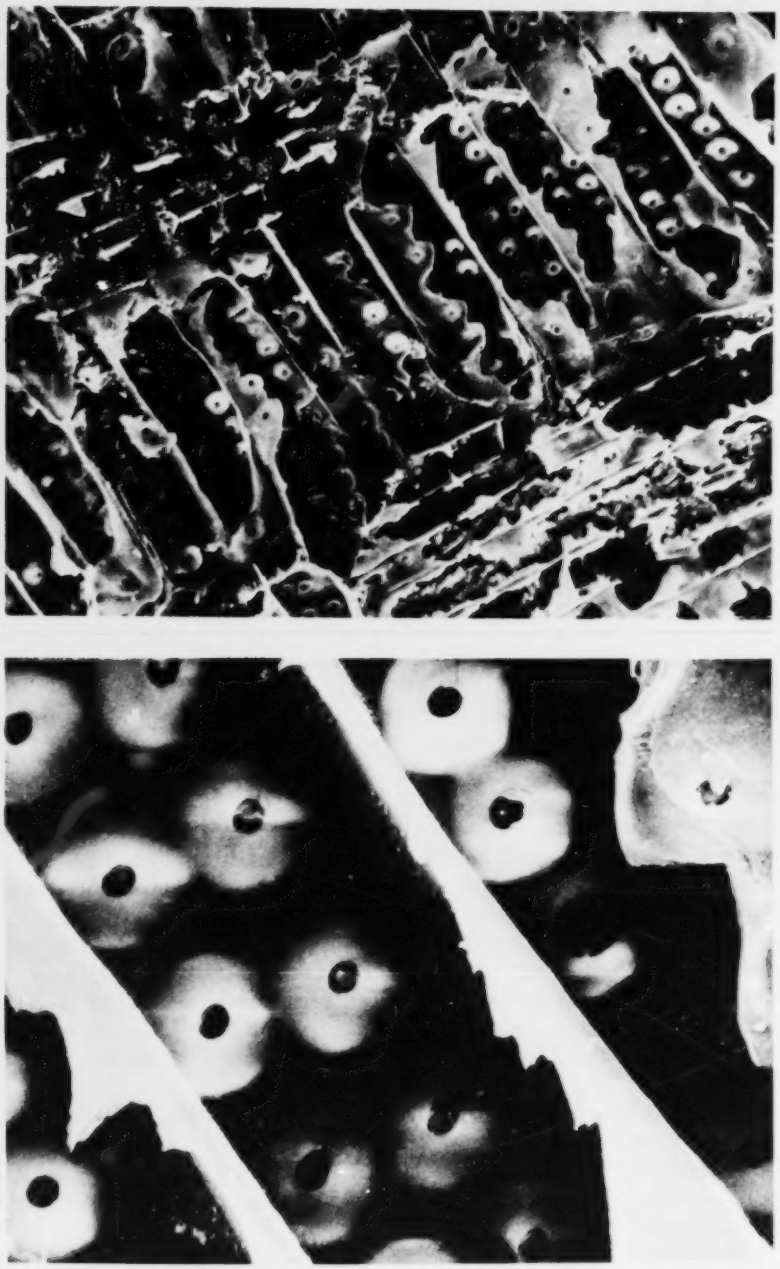


Figure 10 (top). Scanning electron micrograph (SEM) of interior of redwood wide ring count sapwood (200X). Trachael tubes with round bordered pits are shown. Arrow indicates field enlarged for Figure 2.

Figure 11 (bottom). SEM of the interior of a trachael tube showing bacteria present (1,000X).

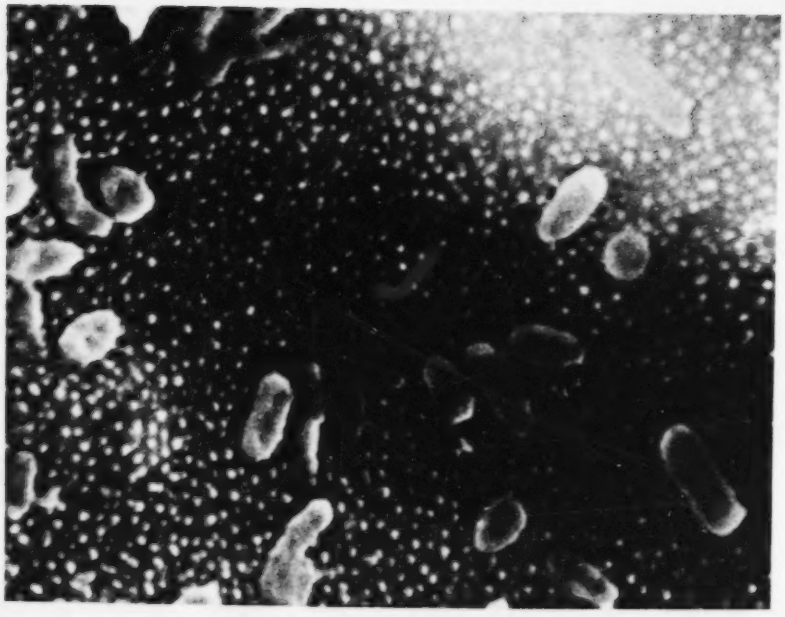


Figure 12. Morphology of individual bacteria within a trachael tube (5,000X).

Although large numbers of total coliforms were isolated from redwood lumber, and to a lesser degree from other woods, none of the isolates were fecal coliform positive. It has been previously illustrated that fecal coliform positive Klebsiella have been isolated from redwood sawdust, from small, experimental redwood tanks, and from public drinking water emanating from redwood reservoirs. While the absence of fecal coliform positive isolates might lead some to believe these coliforms in lumber are of non-fecal origins, the comments concerning the health significance of different Klebsiella biotypes/species mentioned earlier, must also be considered.

In summary, coliforms in drinking water emanating from redwood reservoirs arise from at least two general types of contamination. This includes the colonization of redwood lumber before tank assembly and contamination arising from unsatisfactory source or distribution water after tank assembly.

Evidence of wood contamination prior to tank assembly was demonstrated by the presence of large numbers of coliforms present inside of freshly cut redwood. This contamination could have been caused by the activity of wood-boring insects, birds, and the exposure of lumber to the elements before and after processing at the mill. Because of the unique growth promoting water soluble nutrients in redwood (see next section), any low

level of contamination by Klebsiella during storage of staves out of doors or during tank assembly is also likely to allow colonization of stave surfaces (Figs. 2 and 3). Colonization is facilitated by an ineffective chlorine residual.

Fecal coliform positive K. pneumoniae and fecal coliform negative K. oxytoca have been isolated from drinking water emanating from redwood reservoirs (6). These species were characterized in depth in this study and were found to be indistinguishable from isolates derived as primary pathogens from humans and animals. However, since regrowth of Klebsiella occurs in the redwood environment, their presence should not imply that enteric pathogens as Salmonella are also present. The greatest potential health risk therefore involves the simultaneous presence of both Klebsiella and E. coli, indicating that fecal contamination has been recent. Since it is not practical to identify coliforms to species during routine water quality monitoring, coliforms emanating from redwood reservoir water should be monitored and controlled as in any other potable water supply system.

NUTRITIONAL BASIS FOR KLEBSIELLEAE SELECTION IN REDWOOD WATER STORAGE RESERVOIRS

The presence of Klebsielleae in high cell densities in lumber and in drinking water supplies emanating from redwood reservoirs begs the question of the mechanism which supports their presence and promotes their selective proliferation in this unique botanical environment. The report of Anderson (40) illustrates that seven classes of chemicals are water extractable from redwood. In the present studies, two of the classes of compounds were found to be utilized by Klebsielleae as a source of fermentable carbon. These compounds are cyclitols and free sugars.

It was reported that 4.5% of the aqueous extracts of dry redwood were cyclitols and only 0.1% were free sugars (40). The sugars present are L-arabinose, D-glucose, D-fructose, L-rhamnose, sucrose, and raffinose. A review of the biochemical reactions of the Enterobacteriaceae indicates that representatives of all the genera are able to utilize one or more of these sugars as an energy source (2). Thus, carbohydrates could not be assumed to represent the selective basis for Klebsiella and Enterobacter multiplication in redwood environments.

The cyclitols present in redwood extracts consist of pinitol (60%), sequoyitol (30%), and inositol (10%) [Fig. 13, references 17,40)]. A review of the biochemical reactions of the Enterobacteriaceae indicates that inositol is a distinct differential substrate fermented almost solely by members of the Klebsielleae (2). Information on the fermentation of the other cyclitols by Enterobacteriaceae was not found in the literature.

Several approaches were used to determine the nutritional significance of cyclitols in selectively supporting the multiplication of Klebsiella and Enterobacter in drinking water present in redwood reservoirs. These studies

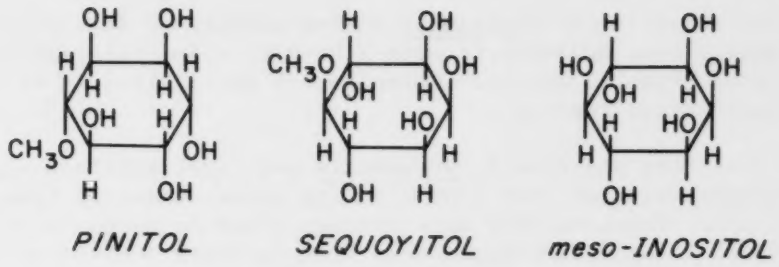


Figure 13. Cyclitols present in aqueous redwood extracts.

included surveys of the fermentability of cyclitols by various genera of enteric bacteria, turbidimetric measurement of growth in defined media containing a cyclitol as the sole carbon and energy source, measuring growth in aqueous extracts of redwood sawdust, and finally, the in situ measurements of cyclitol utilization in aqueous redwood extracts as determined by gas chromatography.

The data in Tables 17 and 18 illustrate the genera of various enteric bacteria which produce acid in phenol red broth base containing a cyclitol as the fermentable substrate. Some 180 isolates of the Tribe Klebsielleae were tested for inositol fermentation and only 1 isolate of Enterobacter cloacae was negative. Over 80% of the Klebsiella isolates from both clinical and non-clinical environments also fermented pinitol while fewer isolates of Enterobacter and neither of the Serratia possessed this capacity. None of the E. coli, Aeromonas, Shigella, and Proteus isolates fermented any of the cyclitols tested. A smaller number of isolates was tested with sequoyitol because of the small amount of this chemical available for testing. Nevertheless, 37/40 members of the Klebsielleae also fermented this cyclitol. Some isolates of Salmonella and Yersinia enterocolitica also fermented 1 or more cyclitols and thus there exists the possibility that enteric pathogens may be capable of colonizing the redwood environment as well. Results of further examinations in the other test systems, however, illustrate that this is not to be expected in the case of Salmonella. Also, exhaustive attempts to isolate Salmonella by conventional enrichment procedures from redwood sawdust, lumber, and from the 3,785 liter (1,000 gal) experimental tank failed. Isolation of Yersinia was not attempted.

In order to confirm that cyclitols can serve as sole sources of carbon and energy, selected enteric bacteria were grown in a defined medium with either 0.2% inositol or pinitol. Growth at 35°C was monitored by measuring culture turbidity. Table 19 indicates that all tested Klebsiella, Entero-

bacter, Serratia, and Erwinia exhibited growth to moderately high levels of turbidity. The E. coli, P. aeruginosa, and Citrobacter clearly did not grow while the Yersinia isolate exhibited less than 2 generations of growth. Figs. 14 and 15 demonstrate the growth kinetics of 4 selected Klebsiella while using inositol or pinitol as a carbon and energy source. The growth rate and final cell yield are seen to vary from isolate to isolate, but clearly all Klebsiella tested can utilize either substrate. Control flasks without added cyclitols had insufficient turbidity to measure.

Tables 20 and 21 summarize results of a survey in which various species of enteric bacteria and P. aeruginosa were tested for multiplication in non-sterile (indigenous flora) and sterile aqueous extracts of sawdust (1.67g sawdust/1 water). Twelve of the 14 Klebsiella tested in the sterile extract increased from 10^2 - 10^4 times over the level of inoculation. Nine of the 14 same isolates increased 10^1 - 10^3 times in the presence of indigenous bacteria associated with the sawdust. Other representatives of the Klebsiella and Erwinia also multiplied extensively in the extracts. P. aeruginosa, E. coli, Citrobacter, and Yersinia enterocolitica also multiplied in the sterile extract but, except for modest growth by Yersinia, did not grow in the presence of the indigenous flora. The growth by the latter genus in the sterile extract probably reflects utilization of the low level of carbohydrates present which are likely to also be rapidly metabolized by the indigenous flora.

Except for one significant exception, Salmonella, growth in non-sterile sawdust extracts coincides well with fermentation of cyclitols in phenol red broth base. The lack of Salmonella growth in extracts and the ability to ferment cyclitols in broth reflects the sensitivity of this enteric pathogen to some growth inhibitory substances in aqueous sawdust extracts. Klebsiella and Erwinia are resistant to such inhibition.

In the final experimental series a gas chromatograph was utilized to detect cyclitols in redwood reservoir water and to demonstrate the in situ utilization of these nutrients by Klebsiella and Enterobacter in aqueous redwood extracts.

Fig. 16 demonstrates a typical gas chromatograph profile and retention times for components contained in a 4 g/1 aqueous redwood sawdust extract. All cyclitols present in redwood are detected in the relative proportions reported previously (40), and are sufficiently resolved so as to permit quantitative estimates of their concentration.

In all growth experiments an extract was prepared from 1.67 g sawdust/1 distilled water even though inositol could not be detected in this more dilute preparation (Fig. 17).

This extract was a compromise which reflected a sawdust extract cyclitol concentration comparable to what was observed in water stored in redwood reservoirs and which was neither growth inhibitory due to high polyphenolic concentrations nor provided sufficient extraneous contaminants to indiscriminatly allow the growth of non-cyclitol using

TABLE 17. UTILIZATION OF INOSITOL AND PINITOL BY ENTEROBACTERIACEAE AND PSEUDOMONAS

	Number Tested	Positive On Phenol Red Inositol (1%) Number Positive	%	Positive On Phenol Red Pinitol (1%) Number Positive	%
Escherichia coli	26	0	0	0	0
Shigella	3	0	0	0	0
Salmonella	5	2	40	2	40
Citrobacter	2	1	50	0	0
Klebsiella pneumoniae (ATCC)	3	3	100	3	100
Klebsiella pneumoniae (environ.)	60	60	100	52	87
Klebsiella pneumoniae (clinical)	43	43	100	39	91
Klebsiella pneumoniae (mastitis)	30	30	100	23	77
Klebsiella (ATCC-oxytoca)	2	not tested		2	100
Enterobacter aerogenes	10	10	100	9	90
Enterobacter agglomerans	21	21	100	11	52
Enterobacter liquefaciens	2	2	100	2	100
Enterobacter hafniae	1	1	100	0	0
Enterobacter cloacae	8	7	88	1	13
Serratia marcescens	2	2	100	0	0
Proteus vulgaris	2	0	0	0	0
Erwinia carotovora	2	1	50	0	0
Erwinia (other)	5	1	20	0	0

(continued)

TABLE 17. (continued)

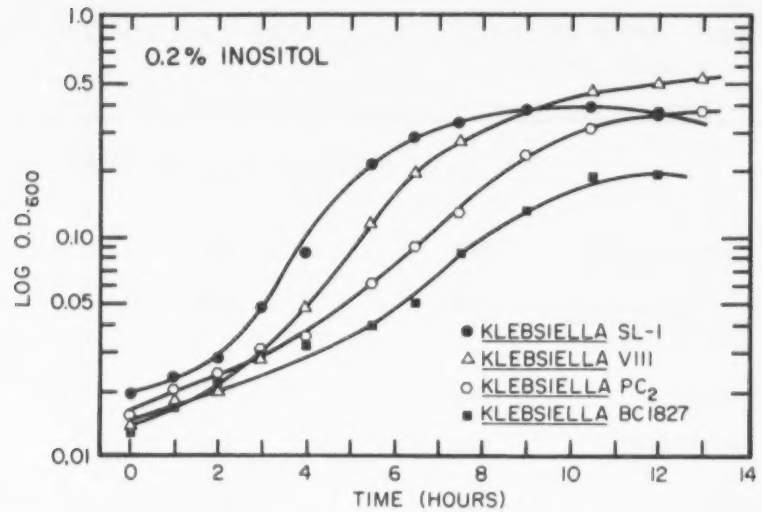
	Number Tested	Positive On Phenol Red Inositol (1%) Number Positive		Positive On Phenol Red Pinitol (1%) Number Positive	
Pseudomonas	3	1	33	0	0
Yersinia enterocolitica	7	7	100	0	0
Yersinia pseudotuberculosis	2	0	0	0	0

TABLE 18. UTILIZATION OF SEQUOYITOL BY ENTEROBACTERIACEAE AND PSEUDOMONAS

	Number Tested	Number Positive	% Positive
Escherichia coli	4	0	0
Shigella	2	0	0
Salmonella	4	2	50
Citrobacter	1	0	0
Klebsiella pneumoniae (ATCC)	3	3	100
Klebsiella pneumoniae (environmental)	18	17	94
Klebsiella pneumoniae (clinical)	7	7	100
Klebsiella pneumoniae (mastitis)	5	5	100
Klebsiella (ATCC-oxytoca)	2	2	100
Enterobacter aerogenes	1	1	100
Enterobacter cloacae	1	0	0
Serratia liquefaciens	1	1	100
Serratia marcescens	2	1	50
Proteus	1	0	0
Erwinia	4	0	0
Yersinia enterocolitica	4	1	25
Pseudomonas	1	0	0

TABLE 19. GROWTH RESPONSE ($O.D._{600}$) WITH 0.2% INOSITOL PLUS BASAL SALTS MEDIUM

Culture	$O.D._{600}$ at 0 hr	$O.D._{600}$ at 13 hr	$O.D._{600}$ at 20 hr
Yersinia enterocolitic	.009	.021	.032
Enterobacter agglomerans (*Erwinia herbicola*)	.009	.039	.112
Erwinia carotovora	.010	.040	.109
Klebsiella ATCC13182	.010	.320	.396
Ps. aeruginosa	.015	.015	.012
Serratia marcescens	.012	.050	.105
Enterobacter aerogenes	.012	.368	.568
E. coli	.009	.011	.009
Citrobacter freundii	.009	.005	.001
Serratia liquefaciens (formerly *Enterobacter*)	.009	.200	.288



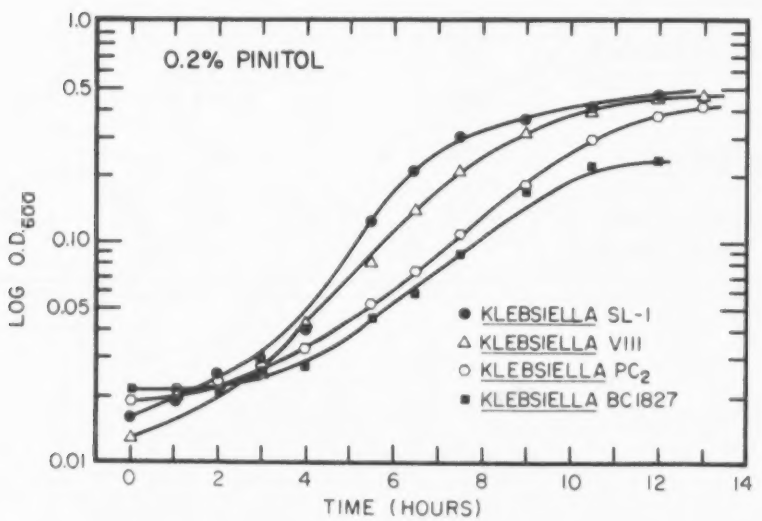


Figure 14 (top). Growth of Klebsiella in defined basal salts medium with inositol as sole carbon and energy source.

Figure 15 (bottom). Growth of Klebsiella in a defined basal salts medium with pinitol as sole carbon and energy source.

TABLE 20. GROWTH RESPONSE IN AQUEOUS EXTRACTS OF REDWOOD SAWDUST IN PRESENCE OF INDIGENOUS FLORA

Culture	Origin	Standard Plate Count per ml, Days After Inoculation		
		0	2	6
Klebsiella pneumoniae				
PSB49-1	bovine masitits	3.9×10^2	4.3×10^4	1.4×10^5
PS401LR	bovine mastitis	2.6×10^2	9.4×10^4	3.0×10^5
MSU1684	bovine mastitis	1.1×10^2	1.1×10^2	3.0×10^1
SL-1	human medical	3.0×10^2	4.7×10^3	3.4×10^4
UT1500	human medical	4.5×10^2	2.1×10^3	3.3×10^3
UO10994	human medical	3.1×10^2	5.6×10^2	3.6×10^3
UO11065	human medical	1.6×10^2	1.8×10^2	1.7×10^2
MH31	human medical	3.9×10^2	6.0×10^2	5.3×10^2
$JH_4 2$	drinking water	6.0×10^1	1.8×10^3	3.0×10^4
$PC_2 II$	drinking water	4.9×10^2	1.5×10^4	7.0×10^5
K_2	redwood	3.0×10^2	3.4×10^2	4.1×10^2
13882	ATCC	4.3×10^2	7.4×10^2	3.2×10^5
13182	ATCC	1.8×10^2	3.4×10^3	1.6×10^4
13883	ATCC	1.6×10^2	5.5×10^2	9.2×10^2
Enterobacter aerogenes 13048	ATCC	1.9×10^2	3.7×10^2	1.1×10^3
Enterobacter cloacae	OSU	3.3×10^2	3.7×10^2	2.1×10^2
Serratia liquefaciens 14460	ATCC	6.5×10^2	7.0×10^3	7.3×10^3
Serratia marcescens	OSU	2.7×10^2	3.8×10^2	4.1×10^2
Enterobacter agglomerans		1.8×10^2	3.2×10^3	3.1×10^5

(continued)

TABLE 20. (continued)

Culture	Origin	Standard Plate Count per ml, Days After Inoculation 0	2	6
Erwinia carotovora		3.0×10^2	1.0×10^5	1.5×10^4
Yersinia enterocolitica		3.0×10^2	1.1×10^4	5.3×10^3
Pseudomonas aeruginosa		5.4×10^2	2.2×10^2	1.3×10^2
Citrobacter freundii		5.9×10^2	5.5×10^2	2.5×10^2
Escherichia coli DS5-1		4.5×10^2	5.0×10^2	1.8×10^2
Shigella dysenteriae		3.2×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Salmonella typhi		1.6×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Salmonella enteritidis		6.6×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Proteus vulgaris		3.1×10^2	2.0×10^2	$<1.0 \times 10^1$
Indigenous flora (uninoculated)		4.6×10^2	1.9×10^5	6.0×10^6
Water control (PSB49-1)		5.4×10^2	5.0×10^2	5.8×10^2

TABLE 21. GROWTH RESPONSE IN STERILE EXTRACTS OF REDWOOD SAWDUST

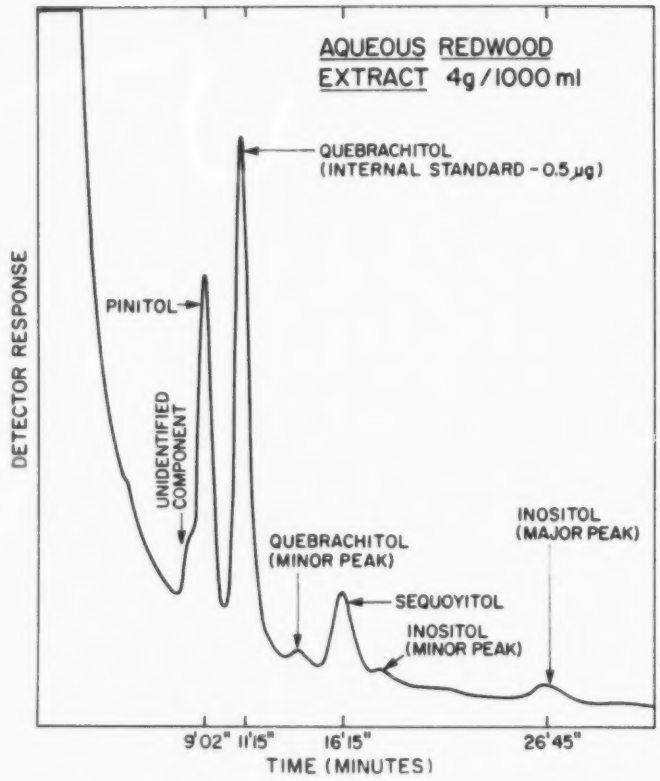
Culture	Standard Plate count per ml, days after inoculation		
Klebsiella pneumoniae	0	2	6
PSB-49-1	4.0×10^2	6.7×10^3	1.1×10^6
PS 401LR	6.4×10^2	2.1×10^4	1.1×10^6
MSU 1684	1.8×10^2	9.0×10^1	2.0×10^1
SL 1	7.4×10^2	4.7×10^3	1.1×10^6
UT 1500	4.1×10^2	3.0×10^4	1.5×10^6
UO 10994	6.6×10^2	5.8×10^2	8.7×10^4
UO 11065	6.6×10^2	9.5×10^2	6.8×10^4
MH 31	7.3×10^2	1.8×10^4	1.2×10^6
$JH_4 2$	4.9×10^2	1.3×10^4	5.6×10^5
$PC_2 II$	1.1×10^3	4.3×10^4	1.3×10^6
K_2	5.2×10^2	6.7×10^3	3.4×10^6
13882	8.8×10^2	1.0×10^3	1.1×10^6
13182	6.4×10^2	8.8×10^2	1.1×10^6
13883	5.4×10^2	1.3×10^3	2.1×10^4
Enterobacter aerogenes 13048	1.0×10^3	1.2×10^3	1.5×10^5
Enterobacter cloacae	3.6×10^2	6.1×10^2	8.0×10^2
Enterobacter agglomerans	1.7×10^2	1.2×10^2	3.7×10^5
Serratia liquefaciens 14460	3.1×10^2	1.7×10^3	1.2×10^6
Serratia marcescens	2.5×10^2	3.1×10^2	8.9×10^2

(continued)

TABLE 21. (continued)

Culture	Standard Plate count per ml, days after inoculation		
	0	2	6
Erwinia carotovora	1.1×10^3	2.3×10^4	2.5×10^6
Yersinia enterocolitica 867	5.5×10^2	3.1×10^2	2.4×10^2
Pseudomonas aeruginosa	1.5×10^3	1.8×10^2	5.9×10^5
Citrobacter freundii	9.5×10^2	6.6×10^2	1.1×10^4
Escherichia coli DS5-1	5.6×10^2	7.6×10^2	3.3×10^3
Shigella dysenteriae	1.5×10^2	1.1×10^2	1.0×10^1
Salmonella enteritidis ser. paratyphi B	8.5×10^2	3.0×10^1	1.0×10^1
	1.6×10^3	1.2×10^3	1.0×10^1

Procedure for these experiments was the same as with the non-sterile redwood (Table 20), except the redwood extract was filter sterilized before it was placed in the flasks, and the cultures were plated on plate count agar without antibotics.



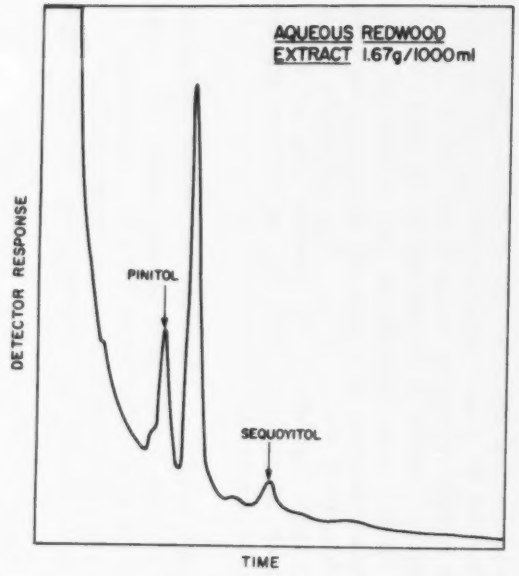


Figure 16. Gas chromatograph (GC) profile of an aqueous redwood sawdust extract illustrating the relative abundance of the 3 cyclitols present.

Figure 17. GC profile of an aqueous redwood sawdust extract at the optimum concentration used to measure in situ utilization of cyclitols (see Figs. 21-23).

species (Table 22a). Figures 18, 19 and 20 show the relative proportion of cyclitols present in water stored in small experimental redwood tanks housed in the laboratory. Tank 2 was adjusted to an 8-day retention time and was on line about 2 months (about 8 retention turnovers) when the determinations were made. Tank 3 was sampled on two occasions, the first 1 month and the second 2 months after addition of finished municipal drinking water. The water in this tank was static. The profiles illustrate the continuing accumulation of cyclitols which are leached out of the wooden staves. The concentrations of these cyclitols, which can support growth of Klebsielleae, are presented in Table 23.

Bacterial studies on the tank water at the time of sample preparation showed tank 3 contained about 10^3 total coliforms/ml and some 10^6 total bacterial/ml. Tank 2 had no detectable coliforms (by MF). However, these filters were massively contaminated with non-coliform colonies which could have masked coliform detection and enumeration in both tank samples.

Cyclitols can still be detected in the tank water probably because cyclitols are leached out faster than coliforms can use them under the given temperature and overall nutritional limitations of the milieu. Elevated cyclitol concentrations are more likely to be found in newer redwood reservoirs during the first year or so of operation. Tank 2, which has water continuously entering and leaving, probably established a flora and nutrient concentration much like that operating in a chemostat.

Figs. 21-23 illustrate the cyclitol profiles after 4 days of incubation of the indicated cultures in the 1.67 g/1,000 ml sawdust extract. The profiles demonstrate that during growth to levels of 10^5 to 10^6 cells/ ml there is an obvious decline in both the sequoyitol and pinitol peak heights (control is Fig. 17). Table 24 summarizes the quantitative utilization of these cyclitols during the incubation of Klebsiella and an E. agglomerans isolate which was also examined. The Klebsiella utilized 60-90% of the available pinitol and 90-100% of the sequoyitol while the E. agglomerans used 94-100% of both cyclitols. These results confirm that under conditions which simulate those found in redwood reservoirs, the Klebsiella and Enterobacter are capable of multiplication on cyclitols as a source of carbon and energy. Cyclitols leach from the staves due to their extraction by water. These observations can account for the presence of high numbers of Klebsielleae in redwood lumber, on staves, and in the drinking water stored in redwood reservoirs. This confirms that necessity for maintaining adequate chlorine residuals, short retention times, and adequate mixing of finished drinking water stored in redwood reservoirs.

TABLE 22. GROWTH RESPONSE IN VARIOUS CONCENTRATIONS OF AQUEOUS EXTRACTS OF REDWOOD SAWDUST

	Grams of redwood used per liter of water	Standard plate count per ml, days after inoculation		
		0	2	6
Klebsiella pneumoniae PSB49-1	0	5.5×10^2	4.5×10^2	4.0×10^2
	.10	5.4×10^2	5.1×10^2	3.3×10^2
	.21	3.5×10^2	3.7×10^2	1.3×10^3
	.42	4.0×10^2	7.5×10^4	8.2×10^5
	.835	4.2×10^2	1.0×10^4	5.3×10^5
	1.67	6.2×10^2	2.1×10^4	8.4×10^5
	8.35	4.2×10^2	6.8×10^4	1.3×10^5
Escherichia coli DS5-1	0	6.0×10^2	4.9×10^2	2.1×10^2
	.10	6.3×10^2	5.6×10^2	3.4×10^2
	.21	5.8×10^2	5.8×10^2	3.6×10^2
	.42	4.5×10^2	5.0×10^2	1.8×10^2
	.835	5.8×10^2	4.1×10^2	9.0×10^1
	1.67	6.2×10^2	7.0×10^2	6.8×10^1
	8.35	4.6×10^2	1.8×10^2	6.0×10^1

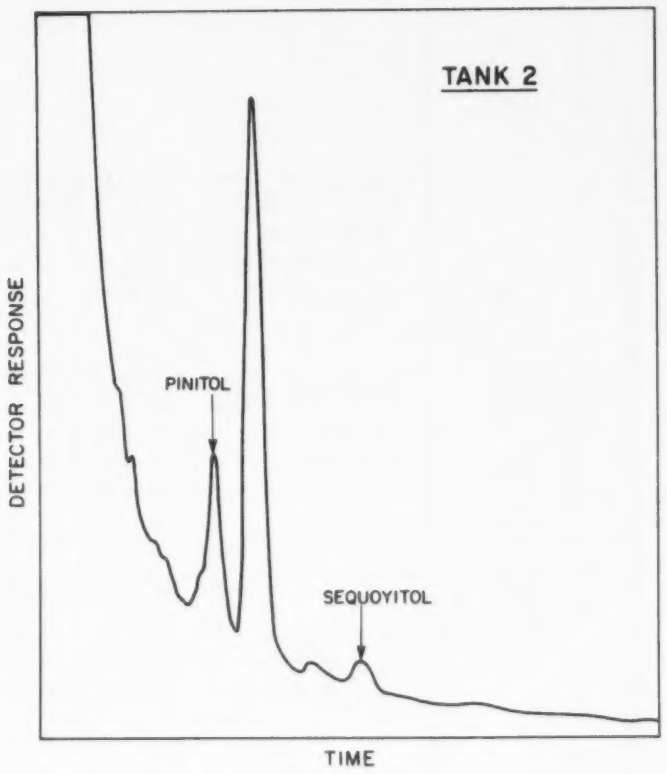
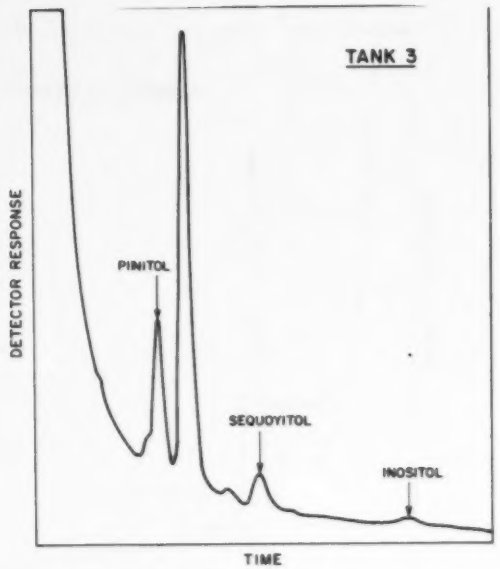


Figure 18. Cyclitols present in redwood tank water on 8 day retention period.



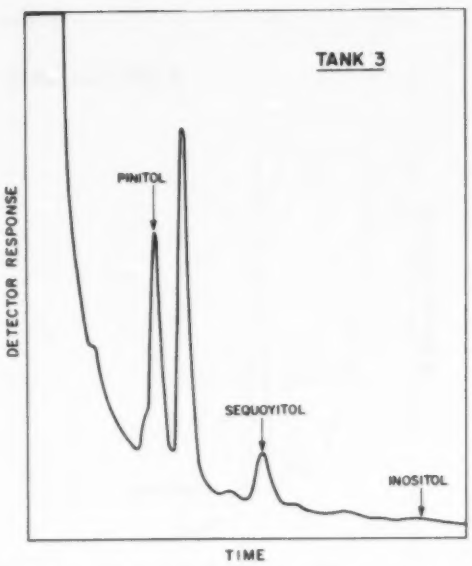
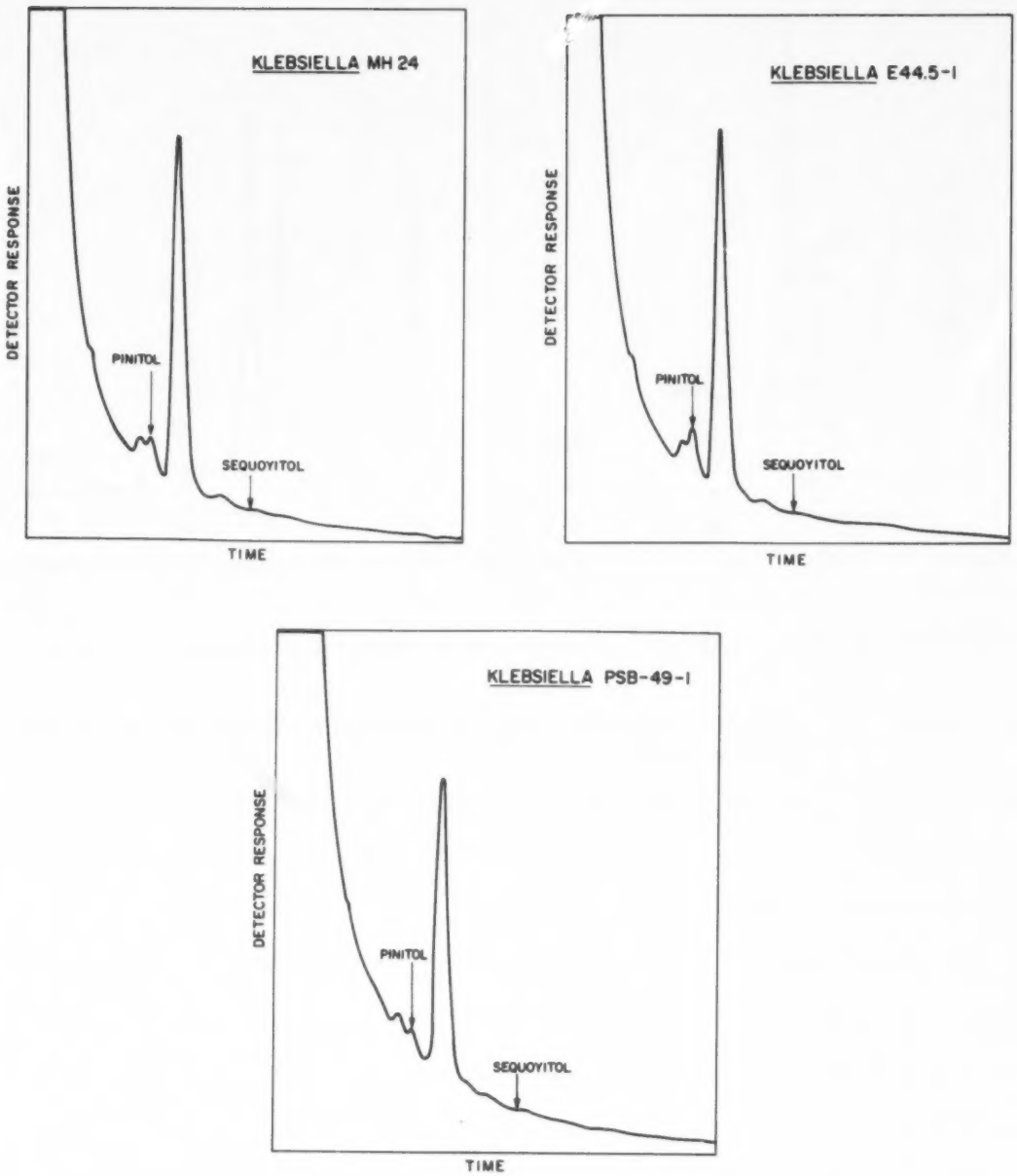


Figure 19 (left). Cyclitols present in redwood tank water held stagnant for 1 month.

Figure 20 (right). Cyclitols present when water is stagnant for 2 months.

TABLE 23. CYCLITOL CONCENTRATIONS IN REDWOOD EXTRACTS AND EXPERIMENTAL REDWOOD TANKS

SAMPLE	ug/ml pinitol	ug/ml sequoyitol	ug/ml inositol
1.67g sawdust/1000ml	30.38	10.24	ND
4.00g sawdust/1000ml	60.49	19.51	8.36
Tank 3 3/10/78	28.58	10.38	1.95
Tank 3 3/14/78	56.61	18.58	0.35
Tank 2 3/14/78	26.51	5.61	ND



Figures 21-23. Cyclitols remaining in 1.67 g/liter agueous redwood sawdust extract after 4 days of incubation of Klebsiella at 35°C. MH24, human origin; E44.5-1, redwood sawdust origin; PSB-49-1, bovine mastitis origin.

TABLE 24. UTILIZATION OF CYCLITOLS IN REDWOOD EXTRACT BY KLEBSIELLEAE

Culture	Pinitol µg/ml in extract			Sequoyitol µg/ml in extract		
	at inoculation	4 days	% utilization	at inoculation	4 days	% utilization
E. agglomerans (Erwinia herbicola ICPB EH103)	30.4	1.8	94	10.2	ND	100
Klebsiella						
E44.5-1 (redwood)	30.4	9.1	70	10.2	ND	100
MH 24 (vegetable)	30.4	6.5	79	10.2	ND	100
BC 1959 (pulp mill)	30.4	12.6	59	10.2	0.8	92
PSB-49-1 (mastitis)	30.4	3.0	90	10.2	ND	100

ND = not detected.

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APPENDIX

COMPARISON OF STANDARD PLATE COUNT OBTAINED WITH STANDARD METHODS AND TRIS BUFFERS AS DILUENTS

The purpose of these experiments was to compare the efficiency in the recovery of viable counts of various Klebsiella cultures following dilutions through standard methods phosphate buffer (SM) and Tris-hydroxymethyl aminoethane (Tris) buffer. The influence of nutritional and temperature stress was also monitored by holding the cells in the diluent on ice during plating and then at 40°C for 2 hr. The results presented in the table below illustrate that Tris buffer diluent yields counts which are higher (although not significantly) than those obtained with SM buffer.

TABLE A-1. COMPARISON OF STANDARD PLATE COUNTS WITH DIFFERENT BUFFERS

Time[d]	Klebsiella Culture[a]					
	ATCC 13883		PSB-49-1		B14	
(hr)	SMB[b]	TB[c]	SMB	TB	SMB	TB
0	73[e]	76	97	122	37	48
	77	64	85	106	36	38
	87	69	120	136	33	38
Average	79	69	100	121	35	41
2	69	87	96	112	35	33
	84	78	99	117	50	65
	81	100	98	139	32	40
Average	78	88	98	123	39	46

[a] Cultures were shaken in Nutrient Broth with 1% added glucose for 20 hr at 37°C.

[b] Standard Methods Phosphate Buffer, sterile.

[c] 0.01 M Tris Buffer pH 7.5, sterile.

(continued)

TABLE A-1. (continued)

[d] Triplicate plate counts were made at 0 and 2 hours after culture dilutions. All samples and dilutions were kept on ice during actual plating times and in a 40°C incubator between platings.

[e] All counts represent number of colonies on the 10^{-8} Standard Plate Count Agar plates. Statistical analyses of all averages, using Student's t at $\alpha = 0.05$, indicate no significant differences between any of the averages, regardless of time sampled or buffer used. All counts are times 10^{7} cells/ml.